

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
 EC₅₀: 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 FEV₁ in liters, FEV₁ percent predicted, and FEV₁/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 10⁵ 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 (EC₅₀) 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).

TABLE I. Subjects' demographics

	Nonsmokers (n = 11)	Smokers (n = 45)	Patients with COPD (n = 20)
Age (y)	60 ± 4	70 ± 2	67 ± 2
Sex (male/female)	8/3	30/15	11/9
FEV ₁ (L)	2.9 ± 0.3	2.5 ± 0.1	1.7 ± 0.1*†
FVC (L)	3.7 ± 0.3	3.5 ± 0.1	3.1 ± 0.2
FEV ₁ (% predicted)	88 ± 6	93 ± 3	68 ± 2*
FVC (% predicted)	100 ± 8	100 ± 5	96 ± 4
FEV ₁ /FVC ratio	0.7 ± 0.02	0.7 ± 0.01	0.5 ± 0.02*†
Smoking history (pack years)	—	39 ± 3	39 ± 7

Data are presented as means ± SEMs.

FVC, Forced vital capacity.

**P* < .05 versus smokers.

†*P* < .01 versus nonsmokers.

TABLE II. Proportion of apoptotic and necrotic cells in each cell fraction

Percoll gradient (vol/vol)	Cell viability (%)			
	Necrotic	Dead	Live	Apoptotic
10% to 20%	3 ± 3	92 ± 3	3 ± 1	3 ± 0
20% to 30%	3 ± 2	78 ± 2	11 ± 2	3 ± 3
30% to 40%	2 ± 1	21 ± 8*	68 ± 10*	10 ± 1
40% to 50%	1 ± 1	13 ± 2*	71 ± 3*	14 ± 5
50% to 60%	1 ± 0	6 ± 2†‡	86 ± 3†‡	9 ± 0

Macrophages were isolated from the different interfaces of the Percoll gradient, and apoptotic and necrotic markers were assessed by using fluorescence-activated cell sorting. Data are presented as means ± SEMs (n = 5).

**P* < .05 and †*P* < .01 versus 10% to 20% (vol/vol).

‡*P* < .05 versus 20% to 30% (vol/vol).

Expression of cell-surface markers on lung macrophages

Initially, multiparameter flow cytometric analysis was performed on macrophage fractions isolated from lung tissue from nonsmokers. Within the cells isolated from the interface of 30% to 40% (vol/vol) Percoll, more than 90% of the cells were HLA-DR⁺, and of these, greater than 95% were positive for CD14 (Fig 1, green and blue boxes), of which approximately 15% were also CD16⁺ (Fig 1, blue box). Within the CD14⁺CD16⁺ population, greater than 70% stained positively for CD163, and of these, greater than 90% were negative for CD40 (Fig 1, gray boxes). Approximately 6% of the HLA-DR⁺ cells were CD14⁺CD16⁺ (Fig 1, orange box), yet subsequent analysis showed that expression of CD163, CD40, and CD206 followed the same pattern as for the CD14⁺CD16⁺ cells. Within the CD14⁺CD16⁺ macrophage population (Fig 1, blue box), greater than 80% of the cells were CD163⁺; however, regardless of CD163 expression, approximately 80% were CD40⁺. All cells expressed CD206 regardless of other cell-surface receptors present. There were no differences in the pattern of cell-surface receptor expression across the 3 Percoll fractions (Fig 1 and see Figs E4 and E5 in this article's Online Repository at www.jacionline.org).

A similar pattern of cell-surface receptor expression was observed in macrophages from the same fraction isolated from the lung tissue of smokers (Fig 2). In this case HLA-DR⁺ cells were greater than 65% positive for CD14 (Fig 2, green and blue boxes), of which approximately 10% were also CD16⁺ (Fig 2, blue box). Within the CD14⁺CD16⁺ population, approximately

60% stained positively for CD163, and of these, greater than 70% were negative for CD40 (Fig 2, gray boxes). Approximately 30% of the HLA-DR⁺ cells were CD14⁺CD16⁺ (Fig 2, orange box), with approximately half being CD163⁺CD40⁺CD206⁺. The CD14⁺CD16⁺ macrophage population (Fig 2, blue box) were greater than 70% CD163⁺; however, regardless of CD163 expression, approximately 60% were CD40⁺. All cells expressed CD206 (data not shown). Again, no difference in expression of any of the markers examined could be determined across the different macrophage fractions (see Figs E6 and E7 in this article's Online Repository at www.jacionline.org).

Finally, a similar analysis was performed on macrophages isolated from 30% to 40% (vol/vol) Percoll interface from the tissue of patients with COPD. In this instance only approximately 50% were positive for CD14 (Fig 3, green and blue boxes), of which approximately 25% were also CD16⁺ (Fig 3, blue box). Of the CD14⁺CD16⁺ population, approximately 15% were CD163⁺, and of these, all were CD40⁺ (Fig 3, gray box). Approximately half the macrophages were CD14⁺CD16⁺ (Fig 3, orange box), and of these, most were CD163⁺CD40⁺ yet stained positive for CD206 (data not shown). The CD14⁺CD16⁺ macrophage population (Fig 3, blue box) showed an equal distribution of cells positive and negative for CD163. However, regardless of CD163 expression, all were CD40⁺ (data not shown). All cells expressed CD206 (data not shown), and again, there were no differences across the different fractions (see Figs E8 and E9 in this article's Online Repository at www.jacionline.org).

This multiparameter approach suggested that macrophages isolated from the lungs of patients with COPD exhibited a different pattern of marker expression compared with macrophages isolated from the lungs of nonsmokers and smokers. To perform further comparisons, the percentage of HLA-DR⁺ cells and the level of each specific marker, as assessed by using MFI, were also analyzed.

There were no significant differences in the expression of HLA-DR with increasing cell density or between subject groups (data not shown). Fewer lung tissue macrophages isolated from patients with COPD were positive for CD14, CD16, CD40, and CD163 compared with any other subject group, with no difference observed between cell densities (Fig 4, A-D). However, all cells expressed CD206 (Fig 4, E). Macrophages from patients with COPD also expressed lower receptor levels, as measured by using MFI, when compared with cells from nonsmokers and smokers, with no differences observed between cell densities (Fig 4, F-J).

Having shown that macrophages from patients with COPD have altered expression of cell-surface markers and that fractionation on the basis of density did not alter these markers, it was important to determine whether this was associated with macrophage function.

Measurement of inflammatory mediator release from lung tissue macrophages

Alveolar macrophages from patients with COPD are activated, releasing proteases and cytokines that are resistant to glucocorticosteroid inhibition,^{6,31,32} and therefore the functional responses of each of the macrophage fractions from the different subject groups were examined. Macrophages extracted from lung tissue from smokers and patients with COPD released greater amounts of active MMP-9 compared with those from nonsmokers (nonsmokers: 2.8 ± 1.1 ng/mL vs smokers: 19.6 ± 5.2 ng/mL

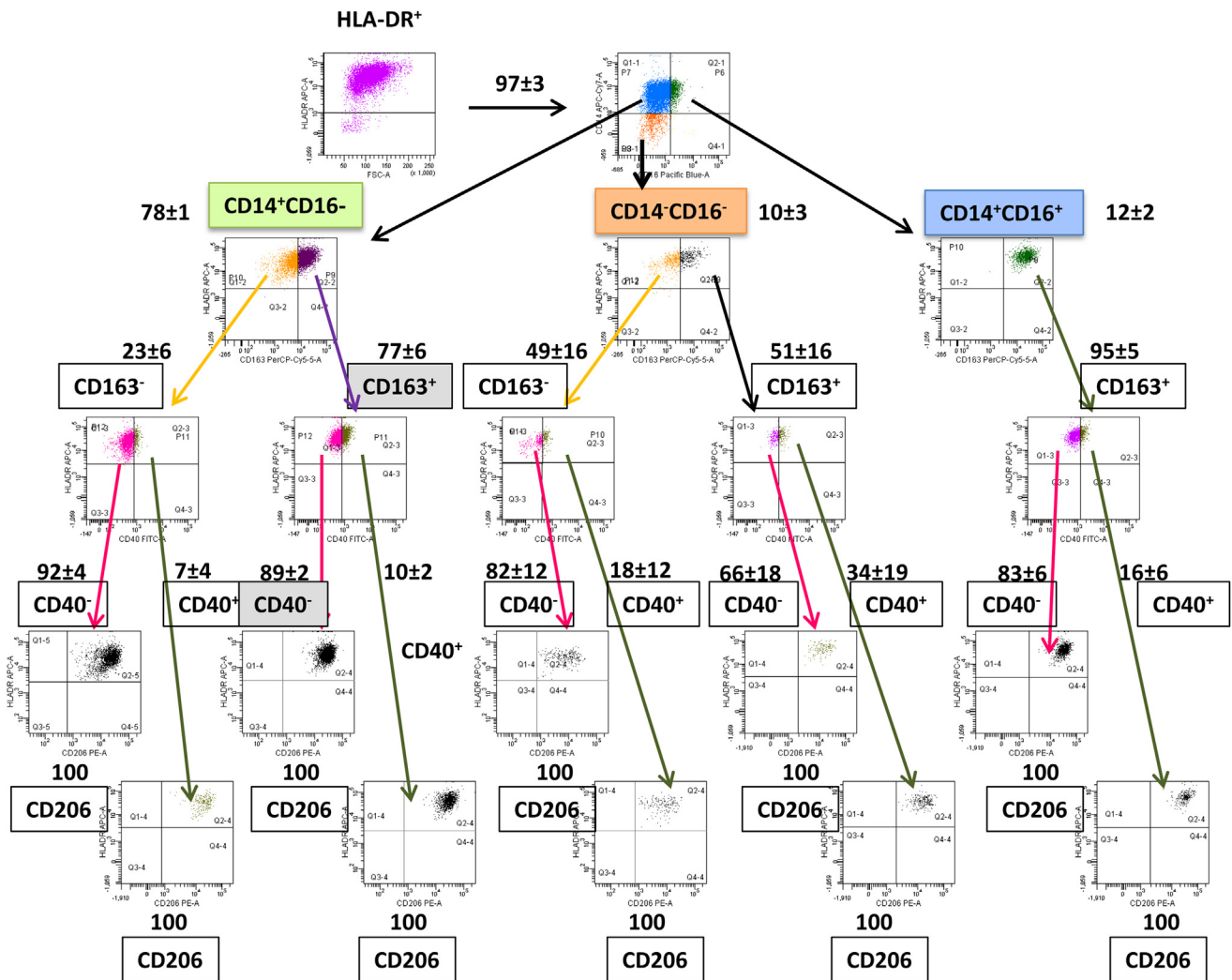


FIG 1. Cell-surface receptor expression of macrophages isolated from the 30% to 40% (vol/vol) Percoll interface from the lung tissue of nonsmokers. Macrophages were isolated at the 30% to 40% (vol/vol) Percoll interface from the lung tissue of nonsmokers, and cells were incubated with APC-HLA-DR, FITC-CD40, PerCP CY5.5-CD163, PE-CD206, APC-CY7-CD14, and Pacific blue-CD16 for 45 minutes at 4°C and analyzed by using flow cytometry. The percentage of cells isolated from the Percoll interfaces of 30% to 40% (vol/vol) are presented as means $\pm$ SEMs ($n = 3$). The figure is representative of 3 experiments.

and vs patients with COPD: 15.2 ± 6.5 ng/mL MMP-9, $P < .05$; Fig 5, A). A similar trend was observed in the denser macrophage fractions, but this was not significant (Fig 5, B and C). These data suggested that the density of macrophages was associated with functional differences of the cells; therefore the effect of stimulating these cells with LPS and measurement of inflammatory cytokine levels was performed.

Baseline releases of TNF- α , CXCL8, and IL-10 were similar for all cell fractions and between macrophages from different subject groups (Table III). LPS stimulated all macrophage fractions to release similar levels of cytokines, with no difference between fractions or subject groups.

Effect of a glucocorticosteroid on LPS-stimulated cytokine release

Next, the effect of a glucocorticosteroid, budesonide, on the release of LPS-stimulated cytokines was evaluated. Budesonide

inhibited LPS-stimulated TNF- α release from the 30% to 40% (vol/vol) cell fraction extracted from the lungs of nonsmokers and smokers by approximately 60% to 80%, with EC_{50} values of 0.5 ± 0.1 nmol/L ($n = 6$) and 2.0 ± 1.2 nmol/L ($n = 11$), respectively. In contrast, macrophages from the 30% to 40% (vol/vol) cell fraction from patients with COPD were unresponsive to budesonide (Fig 6, A). This effect of glucocorticosteroid insensitivity was less marked in cells from patients with COPD isolated at the 40% to 50% (vol/vol) interface (Fig 6, B), with no difference in responsiveness to glucocorticosteroids in the densest fraction (Fig 6, C).

A similar pattern of glucocorticosteroid responsiveness was observed when CXCL8 release was measured. Again, macrophages from nonsmokers and smokers isolated at the 30% to 40% (vol/vol) interface were susceptible to inhibition by budesonide ($EC_{50} = 0.6 \pm 0.1$ nmol/L [$n = 6$] and 1.0 ± 0.3 nmol/L [$n = 11$], respectively; Fig 6, D). This relative glucocorticosteroid insensitivity was lost within the denser macrophage fractions (Fig 6, E and F).

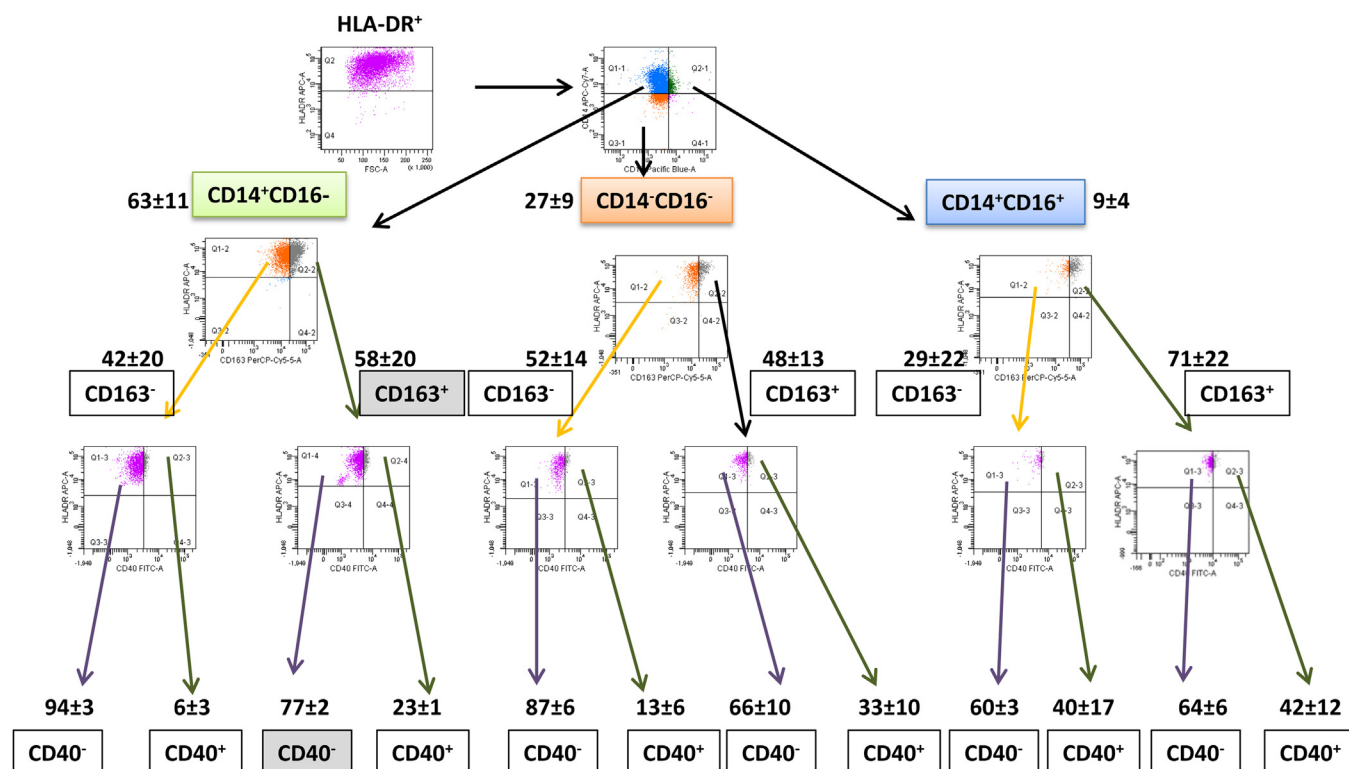


FIG 2. Cell-surface receptor expression of macrophages isolated from the 30% to 40% (vol/vol) Percoll interface from the lung tissue of smokers. Macrophages were isolated at the 30% to 40% (vol/vol) Percoll interface from the lung tissue of nonsmokers, and cells were incubated with APC–HLA-DR, FITC-CD40, PerCP CY5.5–CD163, PE-CD206, APC-CY7–CD14, and Pacific blue–CD16 for 45 minutes at 4°C and analyzed by using flow cytometry. The percentage of cells isolated from the Percoll interfaces of 30% to 40% (vol/vol) are presented as means ± SEMs (n = 3). The figure is representative of 3 experiments.

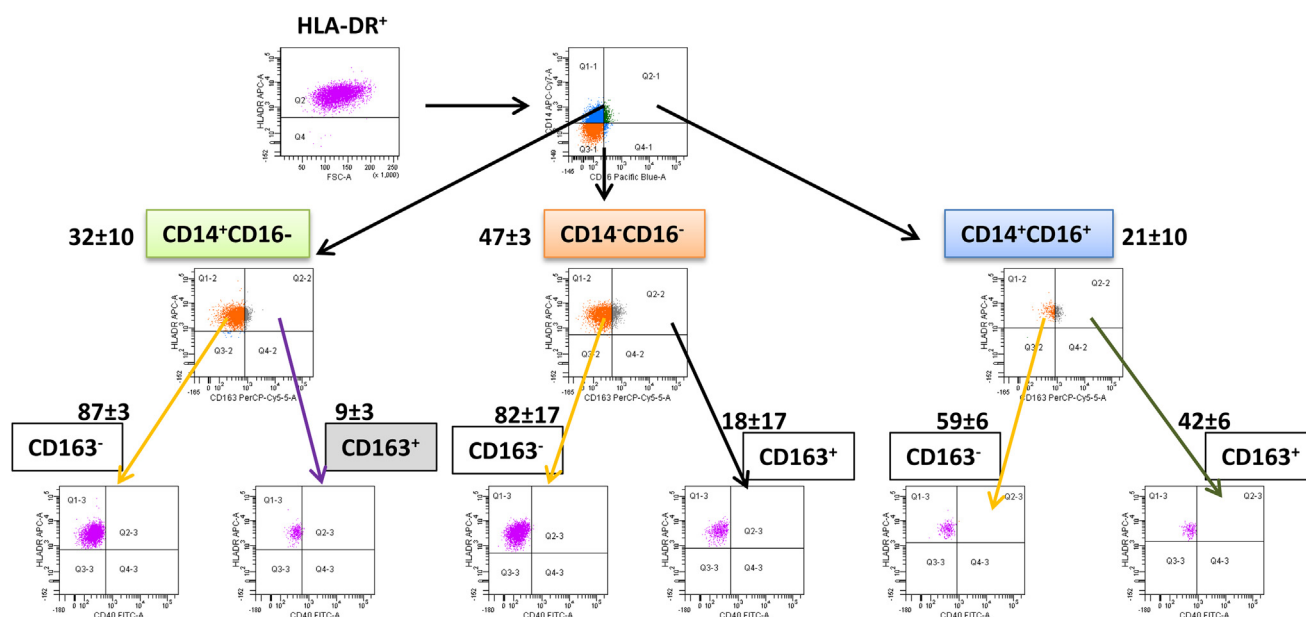


FIG 3. Cell-surface receptor expression of macrophages isolated from the 30% to 40% (vol/vol) Percoll interface from the lung tissue of patients with COPD. Macrophages were isolated at the 30% to 40% (vol/vol) Percoll interface from the lung tissue of nonsmokers, and cells were incubated with APC–HLA-DR, FITC-CD40, PerCP CY5.5–CD163, PE-CD206, APC-CY7–CD14, and Pacific blue–CD16 for 45 minutes at 4°C and analyzed by using flow cytometry. The percentage of cells isolated from the Percoll interfaces of 30% to 40% (vol/vol) are presented as means ± SEMs (n = 3). The figure is representative of 3 experiments.

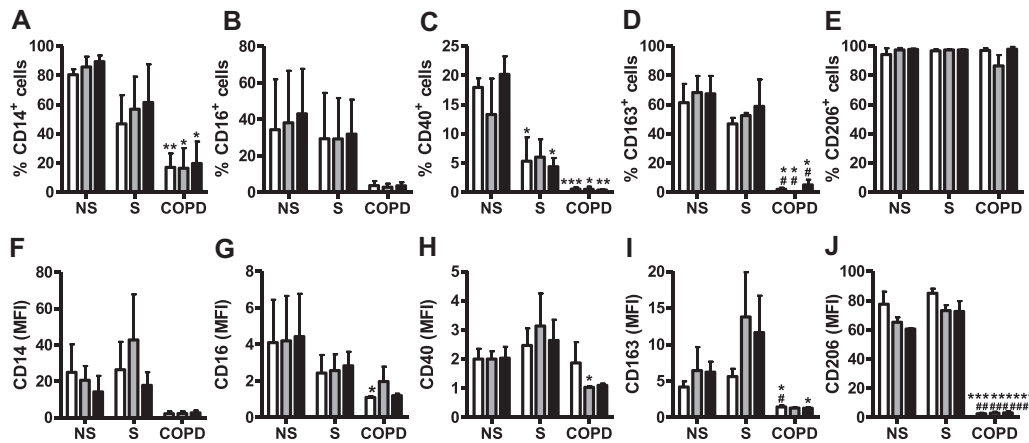


FIG 4. A–J, Expression of cell-surface markers on macrophages isolated from different Percoll interfaces. Macrophages were isolated from resected human lung tissue from nonsmokers (NS; $n = 3$), smokers (S; $n = 3$), and patients with COPD ($n = 3$) at the interfaces of 30% to 40% (vol/vol; open bars), 40% to 50% (vol/vol; gray bars), and 50% to 60% (vol/vol; black bars) Percoll. Cells were blocked with IgG for 15 minutes at room temperature and incubated with the relevant antibody for 45 minutes at 4°C. Cells were washed and resuspended in FACS fix. Data are presented as means $\pm$ SEMs. * $P < .05$, ** $P < .01$, and *** $P < .001$ for differences from nonsmokers. # $P < .05$, ## $P < .01$, and ### $P < .001$ for differences from smokers.

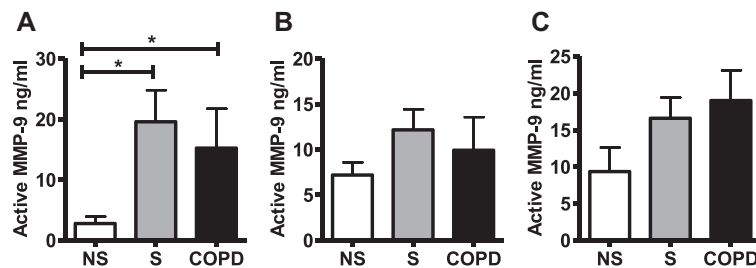


FIG 5. Release of active MMP-9 from lung tissue macrophages. Macrophages were isolated from the 30% to 40% (vol/vol; A), 40% to 50% (vol/vol; B), and 50% to 60% (vol/vol; C) interfaces of the Percoll gradient and seeded into cell-culture plates. MMP-9 release from all cell fractions was measured by using a fluorometric activity assay: nonsmokers (NS; $n = 5$, open bars), smokers (S; $n = 5$, gray bars), and patients with COPD ($n = 3$ –5, black bars). Data are presented as means $\pm$ SEMs. * $P < .05$.

TABLE III. Cytokine release from fractionated human lung macrophages

Percoll gradient (vol/vol)	Baseline (ng/mL)			LPS (10 ng/mL) stimulation (ng/mL)		
	CXCL8	TNF- α	IL-10	CXCL8	TNF- α	IL-10
30% to 40%	34.3 $\pm$ 6.8	1.2 $\pm$ 0.2	0.2 $\pm$ 0.0	160.7 $\pm$ 26.1*	6.2 $\pm$ 1.5§	1.0 $\pm$ 0.3
40% to 50%	35.5 $\pm$ 9.4	1.4 $\pm$ 0.2	0.1 $\pm$ 0.0	168.6 $\pm$ 14.5†	5.6 $\pm$ 1.5‡	1.0 $\pm$ 0.2¶
50% to 60%	23.1 $\pm$ 5.5	1.4 $\pm$ 0.2	0.1 $\pm$ 0.0	131.5 $\pm$ 35.9*	5.7 $\pm$ 1.8‡	0.7 $\pm$ 0.1

Data are presented as means $\pm$ SEMs ($n = 8$).

* $P < .05$ and † $P < .01$ versus CXCL8 baseline.

‡ $P < .05$ and § $P < .01$ versus TNF- α baseline.

|| $P < .05$ and ¶ $P < .01$ versus IL-10 baseline.

Finally, the effect of budesonide on LPS-stimulated IL-10 release was examined. Budesonide inhibited IL-10 release in a concentration-dependent manner from all cell fractions and in all subject groups (Fig 6, G–I). The EC₅₀ values were approximately 1 nmol/L for all cell fractions and subject groups. Maximum LPS-stimulated IL-10 inhibition by budesonide for nonsmokers was approximately 80% and 50% to 60% for smokers and patients with COPD across all fractions (Fig 6, G–I).

DISCUSSION

Macrophages play a pivotal role in the pathophysiology of COPD, in which alveolar macrophages increase in number, release more inflammatory mediators (eg, CXCL8, TNF- α , and MMP-9), and respond less well to glucocorticosteroids.^{6,32} This study investigated the possibility that these macrophages might represent a distinct phenotype associated with COPD and showed that within the pulmonary macrophage population, approximately a third of these cells are glucocorticosteroid

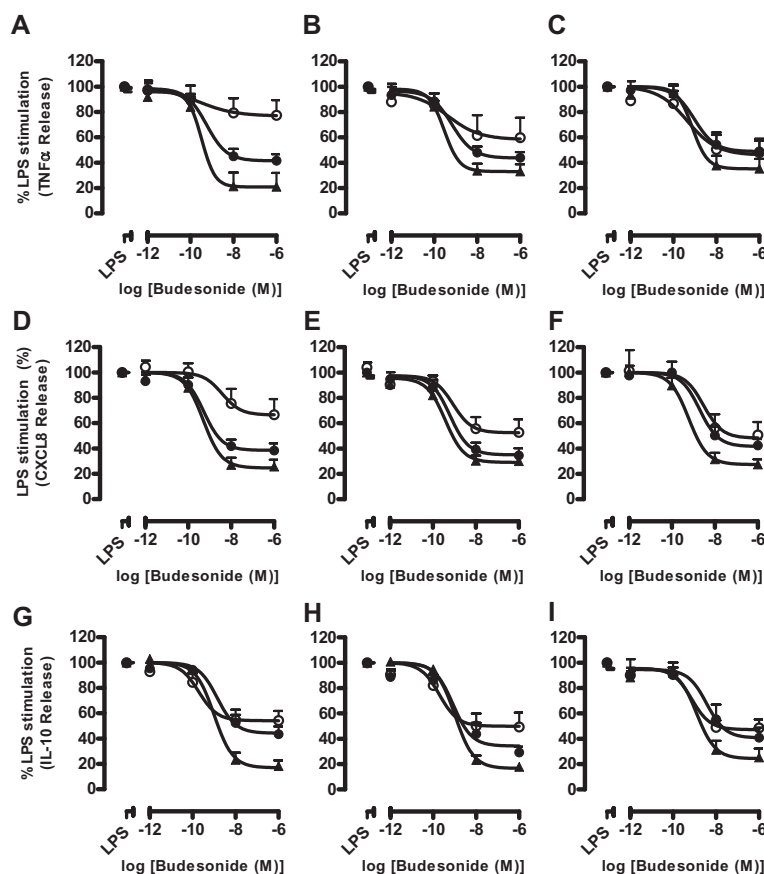


FIG 6. Effect of budesonide on LPS-stimulated cytokine release from lung tissue macrophages. Macrophages were isolated from the 30% to 40% (vol/vol; **A**, **D**, and **G**), 40% to 50% (vol/vol; **B**, **E**, and **H**), and 50% to 60% (vol/vol; **C**, **F**, and **I**) interfaces of the Percoll gradient. Cells were pretreated with budesonide at the concentrations indicated for 1 hour, followed by LPS stimulation (10 ng/mL) for 20 hours, and medium was harvested. TNF- α panels (Fig 6, **A-C**), CXCL8 (Fig 6, **D-F**), and IL-10 (Fig 6, **G-I**) were measured by using ELISA, and data were then normalized to LPS stimulation (100%). Triangles, Nonsmokers ($n = 6$); solid circles, smokers ($n = 11$); open circles, patients with COPD ($n = 7$). Data are presented as means $\pm$ SEMs.

insensitive and release high levels of activated MMP-9 but cannot be distinguished from other macrophages based on the cell-surface markers examined. However, all macrophages from the lungs of patients with COPD had significantly reduced expression of many of the cell-surface receptors.

The present study showed that the least dense macrophage fractions (1.011-1.023 g/mL and 1.023-1.036 g/mL) represented a high proportion of apoptotic/dead fraction cells and were excluded from further analysis. The cell population at the 1.036 to 1.048 g/mL interface (30% to 40% [vol/vol] cell fraction) was similar to that identified in rodents in which cells with a density of 1.036 g/mL appear enlarged and begin to show signs of degeneration,³³ whereas the smaller denser cells (ie, 1.05-1.068 g/mL) appeared intact and showed no signs of degeneration.³⁴ In human subjects with active tuberculosis, alveolar macrophages have a density of less than 1.03 g/mL up to 1.04 g/mL,³⁵ which broadly corresponds to the 30% to 40% (vol/vol) fraction presented here. However, whether macrophage density is a marker of age or of alveolar macrophages in general remains unclear.

It is known that pulmonary macrophages can persist for many years within the lung because donor macrophages can be detected 2 to 3 years after transplantation³⁶; however, specific identification of these long-lived resident cells in a subject who

has not undergone transplantation is difficult. Rodent studies of pulmonary macrophage depletion suggest that macrophages repopulate the lung rapidly,^{37,38} and this turnover is increased after infection.³⁹ These data suggest that during disease processes, macrophage numbers and populations can alter, and therefore similar responses might also be occurring in patients with inflammatory lung diseases, such as COPD.

Cell-surface phenotypic markers were examined with specific reference to macrophages of differing density to determine whether macrophages from the lungs of patients with COPD differed from those isolated from control samples. The multi-parameter flow cytometric methodology is a well-established technique to measure cellular characteristics, but there are many challenges in designing these analyses, including highly variable expression levels of markers, variable brightness of fluorochromes, emission overlap, and stability of some fluorochromes. Furthermore, one of the major hindrances of investigating macrophages using this technique is their intractable autofluorescence. Intracellular autofluorescence of alveolar macrophages is thought to be a marker of endocytosed fluorescent particles from, for example, cigarette smoke.⁴⁰ Furthermore, alveolar macrophage autofluorescence in smokers who cease smoking show reduced autofluorescence.⁴⁰ Although

not investigated specifically in this study, the autofluorescence of macrophages was similar between subject groups investigated, and therefore any differences in fluorescence intensity measured are unlikely to be due to inherent differences in autofluorescence.

Analysis of macrophage cell-surface receptor expression showed greater than 70% of macrophages from nonsmokers and greater than 50% from smokers were CD14⁺CD16[−]CD163⁺ and CD40[−] and therefore appear to display a macrophage phenotype encompassing properties of alternative activation. However, no differences were observed between cells isolated from the different Percoll interfaces, indicating that cell density could not be discriminated by using these markers. Conversely, macrophages isolated from patients with COPD showed a more variable receptor profile. Those that were CD14⁺CD16[−] were greater than 80% CD163[−], and of these, all were CD40[−]. This differs when compared with the cells isolated from the lungs of nonsmokers and smokers. Of the CD14⁺CD16⁺ macrophages isolated from patients with COPD, there was an equal distribution of CD163[−] and CD163⁺ cells, and therefore these could not be characterized as classically or alternatively activated. These patterns were not consistent with a linear change but more of a mix of individual alterations, which were not consistent with a global shift in phenotype.

Examining receptor expression in combination showed a HLA-DR⁺CD14[−]CD16[−] macrophage population was present in all subject groups and cell fractions; this population was most prominent in macrophages isolated from patients with COPD and was distinct from the other macrophage populations. To the best of our knowledge, this population has been identified for the first time in this study and has not previously been reported in the literature. Further analysis was conducted to confirm the data were not being skewed because of high spectral overlap between the fluorochromes for CD14 and CD16 to verify that this population was not an artifact. The fluorochromes associated with CD14 and CD16 were allophycocyanin (APC)–CY7 and Pacific blue, respectively, and as such, there is no spectral overlap. However, the receptors investigated in this study do not show differences between cell fractions. This might be because differences do not exist between the receptor expression levels between cell fractions or the differences between expression levels are too subtle to be detected by using flow cytometry.

Alveolar macrophages from nonsmokers express low CD14 levels, which increase during inflammation.⁴¹ Therefore it would have been expected that macrophages isolated from the lungs of patients with COPD would express higher CD14 levels compared with those seen in control subjects. However, this was not observed but might be due to the lungs of patients with COPD also containing higher levels of neutrophil elastase and other proteases, such as MMP-9,^{42,43} which, in turn, might have removed the receptors from the cell surface. This might also explain why all the receptors investigated were at lower levels on macrophages isolated from the lungs of patients with COPD compared with control subjects and might contribute to the reduced phagocytosis reported for macrophages from patients with COPD.^{44,45}

Alveolar macrophages from patients with COPD release more active MMP-9 than those from nonsmokers and smokers.³² The present study showed that macrophages isolated from the 30% to 40% (vol/vol) Percoll interface from smokers and patients

with COPD released increased levels of active MMP-9 compared with those seen in nonsmokers, suggesting this fraction might correspond to the alveolar macrophage population.³² This difference became less marked with the denser macrophage populations.

A number of studies have indicated that neither high-dose inhaled nor oral corticosteroids reduce the levels of CXCL8 or proteases in induced sputum of patients with COPD.^{5,46,47} This reduced glucocorticosteroid efficacy in patients with COPD could be due to macrophage insensitivity⁶ and is supported by this study in which macrophages isolated from patients with COPD were functionally distinct. The least dense macrophages isolated from the 30% to 40% (vol/vol) Percoll interface were significantly less responsive to budesonide when isolated from patients with COPD compared with cells from tissue of nonsmokers and smokers. This effect was lost with increasing cell density, and all macrophages isolated at the 50% to 60% (vol/vol) interface were sensitive to glucocorticosteroids. These cells might represent a less mature, newly recruited macrophage population. However, glucocorticoid insensitivity in the more mature, less dense cells was selective for LPS-stimulated CXCL8 and TNF- α release because budesonide inhibition of LPS-stimulated IL-10 release from these fractions was similar for macrophages from both smokers and patients with COPD. Although budesonide inhibited proinflammatory responses from the mature macrophage population isolated from patients with COPD, these cells were not completely glucocorticosteroid insensitive because IL-10 was inhibited by this treatment. This suggests that glucocorticosteroids might contribute to the worsening lung inflammation observed in patients with COPD because they do not suppress proinflammatory cytokines but inhibit anti-inflammatory cytokines. The differential steroid responsiveness of each of these fractions might also account for the variability in data reported by other groups.⁴⁸ Therefore macrophages from lung tissue from patients with COPD might represent a phenotype encompassing the properties of both classically activated M1-like and tumor-associated M2b-like macrophages.⁴⁹ Exactly why the more mature macrophages show a differential response to glucocorticosteroids might be due to their residency in a highly oxidative environment. Oxidative stress drives glucocorticosteroid insensitivity,^{7,31,50} and therefore the longer macrophages are exposed to this environment, the more likely they are to develop a reduced response to steroids.

All the lung resection tissue used in this study was macroscopically not tumorous; however, it was predominantly from patients with primary lung cancer. Therefore it is unclear whether tumor-associated cytokines played a role in the macrophage phenotype observed. In addition, the lungs of patients with COPD are colonized with bacteria, such as *Haemophilus influenzae*, which could polarize macrophages toward a classically activated phenotype.⁵¹ This might explain why the macrophages isolated from patients with COPD from the 30% to 40% (vol/vol) Percoll interface present properties of both classically activated and regulatory macrophages.

This study has identified a specific pulmonary macrophage phenotype in patients with COPD that is glucocorticosteroid insensitive. Targeting this population directly might allow future therapies that remove this phenotype specifically so that the host defense role of macrophages is preserved by the remaining populations.

Key messages

- Human pulmonary macrophages from nonsmokers, smokers, or patients with COPD do not align with the M1 and M2 macrophage markers identified by using *in vitro* and *in vivo* models.
- A specific macrophage population with a density of 1.036 to 1.048 g/mL is glucocorticosteroid insensitive in the lungs of patients with COPD.
- Expression of cell-surface markers is reduced in macrophages from patients with COPD, and a HLA-DR⁺CD14⁺CD16⁺ cell type accounts for approximately half of these cells.
- Macrophages from patients with COPD exhibit a functionally distinct phenotype that might be amenable to targeted therapies.

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METHODS

Isolation of lung tissue macrophages

Macrophages were isolated from lung tissue, essentially as described previously.^{E1} Lung tissue was lavaged by injecting 120 mL of macrophage medium (RPMI 1640 containing 100 µg/mL [1% vol/vol] penicillin/streptomycin and 2 mmol/L [1% vol/vol] L-glutamine, 0.25 µg [wt/vol] of amphotericin B, and 50 mmol/L EDTA). The flushed medium was collected and centrifuged at 300g for 5 minutes. The pellet was resuspended in 1 mL of complete macrophage medium (RPMI 1640 containing 10% vol/vol FCS, 100 µg/mL [1% vol/vol] penicillin/streptomycin, 2 mmol/L [1% vol/vol] L-glutamine, and 0.25 µg [wt/vol] amphotericin B) and separated on a Percoll density gradient. One hundred percent (vol/vol) Percoll was derived by adding 3 mL of 10× Dulbecco PBS to 27 mL of Percoll. The gradient consisted of 6 separate concentrations of Percoll prepared as follows: 2 mL of 60% (vol/vol) Percoll was added to a 15-mL Falcon tube. This was overlaid with 2 mL of 50% (vol/vol) Percoll, 2 mL of 40% (vol/vol) Percoll, 2 mL of 30% (vol/vol) Percoll, and 2 mL of 20% (vol/vol) Percoll. The resuspended cell pellet was overlaid onto the prepared gradient and centrifuged at 1000g for 25 minutes. Fractions at each of the Percoll interfaces were collected into individual tubes and washed with 15 mL of sterile PBS and 5 mL of complete medium by means of centrifugation at 300g for 5 minutes. The pellet was resuspended in 10 mL of complete medium, and cells were counted and seeded onto 96-well plates at 10⁵ cells per well. Adherent macrophages were incubated at 37°C, 5% (vol/vol) CO₂ overnight before treatment.

Flow cytometry

BD CompBead Plus Compensation Particles were used to establish the laser voltages before analyzing the cell samples with a BD FACSCanto II

analyzer. For each fluorochrome, the laser voltages were set to provide the best resolution between peaks (ie, between the control, the unstained bead, and the stained bead). As a starting point, the control peak was set to sit at greater than 10⁻² on the x-axis of the histogram; this produced the largest window between the control peak and stained peak. After this had been set for all fluorochromes, the FACSDiva software automatically calculated the amount of spectral overlap between all fluorochromes. If there was an overlap of greater than 35% between any 2 fluorochromes, the voltages were manually altered to reduce the spectral overlap while at the same time maintaining good resolution. Only after these voltages were established were the cells passed through the machine.

Cells were blocked with 1% (vol/vol) human serum for 15 minutes at room temperature and centrifuged at 300g for 5 minutes. HLA-DR-APC (2.5 µg/mL), CD40-fluorescein isothiocyanate (FITC; 12 µg/mL), CD163-peridinin-chlorophyll-protein (PerCP)-CY5.5; (10 µg/mL), CD16-Pacific blue (2 µg/mL), CD14-APC-CY7 (12 µg/mL), CD206-PE (2.5 µg/mL), CD3-PE-Cy7 (4 µg/mL), and CD1A-PE-CY7 were added in combination, and PBS was added to a final volume of 100 µL. Cells were also stained for fluorescence minus one controls. Cells were incubated for 45 minutes at 4°C followed by washes with PBS, centrifuged at 300g for 5 minutes, and resuspended in 400 µL of FACS fix (BD, Franklin Lakes, NJ). The hierarchy of acquisition is shown in Fig E3, with a minimum of 5000 events acquired for each run.

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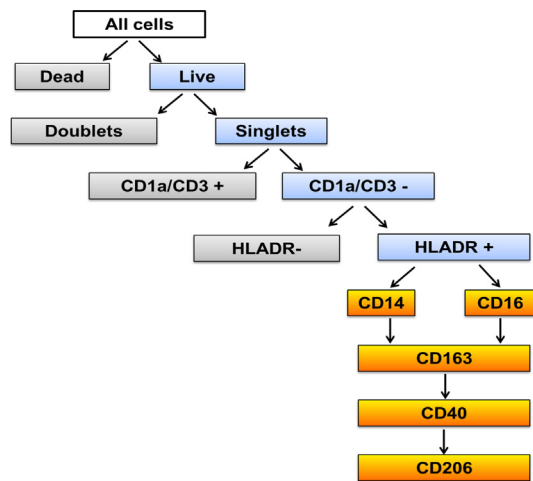


FIG E1. Gating strategy for multiparameter flow cytometry. Dead cells, doublets, and CD3⁺CD1a⁺ cells were excluded, and the remaining HLA-DR⁺ cells were subsequently analyzed for CD14 and CD16, followed by CD163, CD40, and CD206.

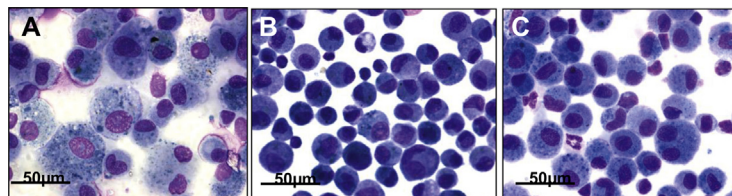


FIG E2. Reastain Quick-Diff staining of tissue macrophages. Nuclei stained *dark blue*, and cytoplasm stained *light blue*. Cell fractions were extracted from the following Percoll interfaces: **A**, 30% to 40% (vol/vol); **B**, 40% to 50% (vol/vol); and **C**, 50% to 60% (vol/vol). Photomicrographs are representative of 6 tissue samples.

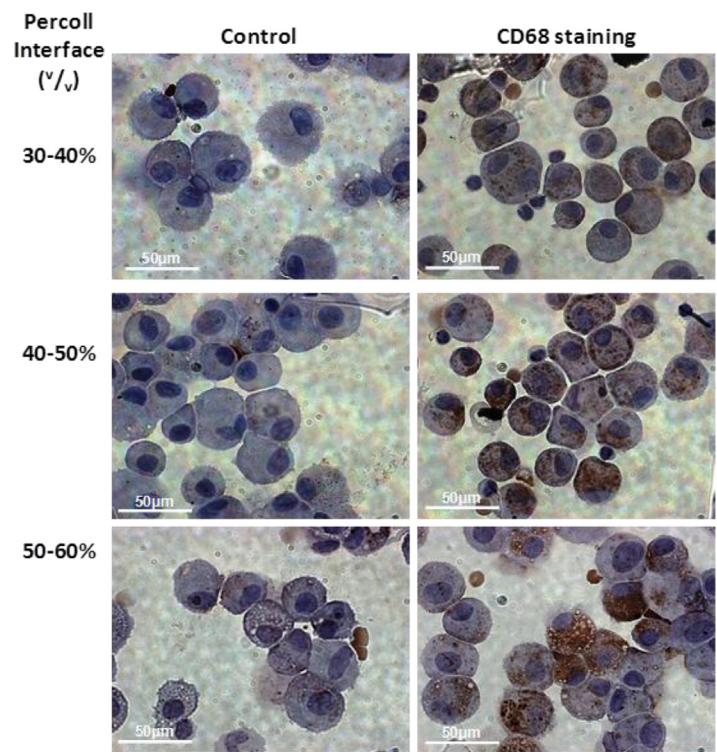


FIG E3. Expression of CD68 by tissue macrophages. Cytospin preparations were prepared with human lung macrophages. Cells were fixed and stained by using an immunocytochemistry kit with either a CD68 antibody or an isotype control. Slides were then visualized with a light microscope. Cellular staining (haemalum) is *purple/blue*, and CD68 staining is *red/brown*. Results are representative of 4 separate experiments.

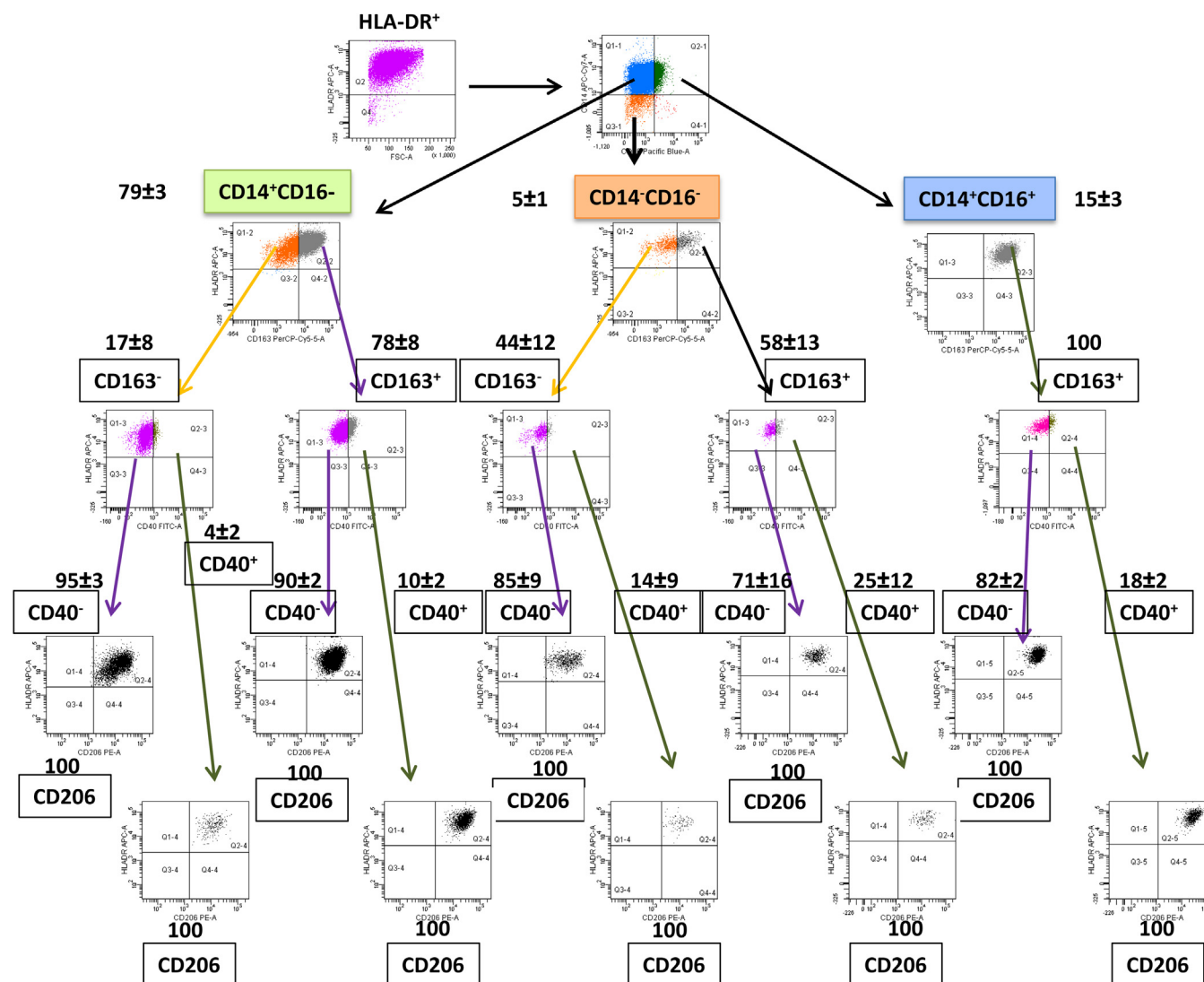
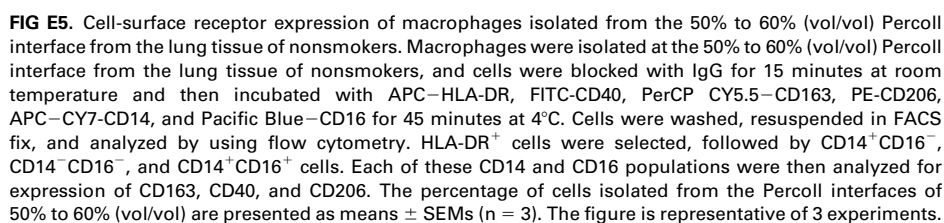


FIG E4. Cell-surface receptor expression of macrophages isolated from the 40% to 50% (vol/vol) Percoll interface from the lung tissue of nonsmokers. Macrophages were isolated at the 40% to 50% (vol/vol) Percoll interface from the lung tissue of nonsmokers, and cells were blocked with IgG for 15 minutes at room temperature and then incubated with APC-HLA-DR, FITC-CD40, PerCP CY5.5-CD163, PE-CD206, APC-CY7-CD14, and Pacific Blue-CD16 for 45 minutes at 4°C. Cells were washed, resuspended in FACS fix, and analyzed by means of flow cytometry. HLA-DR⁺ cells were selected, followed by CD14⁺CD16⁻, CD14⁻CD16⁻, and CD14⁺CD16⁺ cells. Each of these CD14 and CD16 populations were then analyzed for expression of CD163, CD40, and CD206. The percentage of cells isolated from the Percoll interfaces of 40% to 50% (vol/vol) are presented as means ± SEMs (n = 3). The figure is representative of 3 experiments.



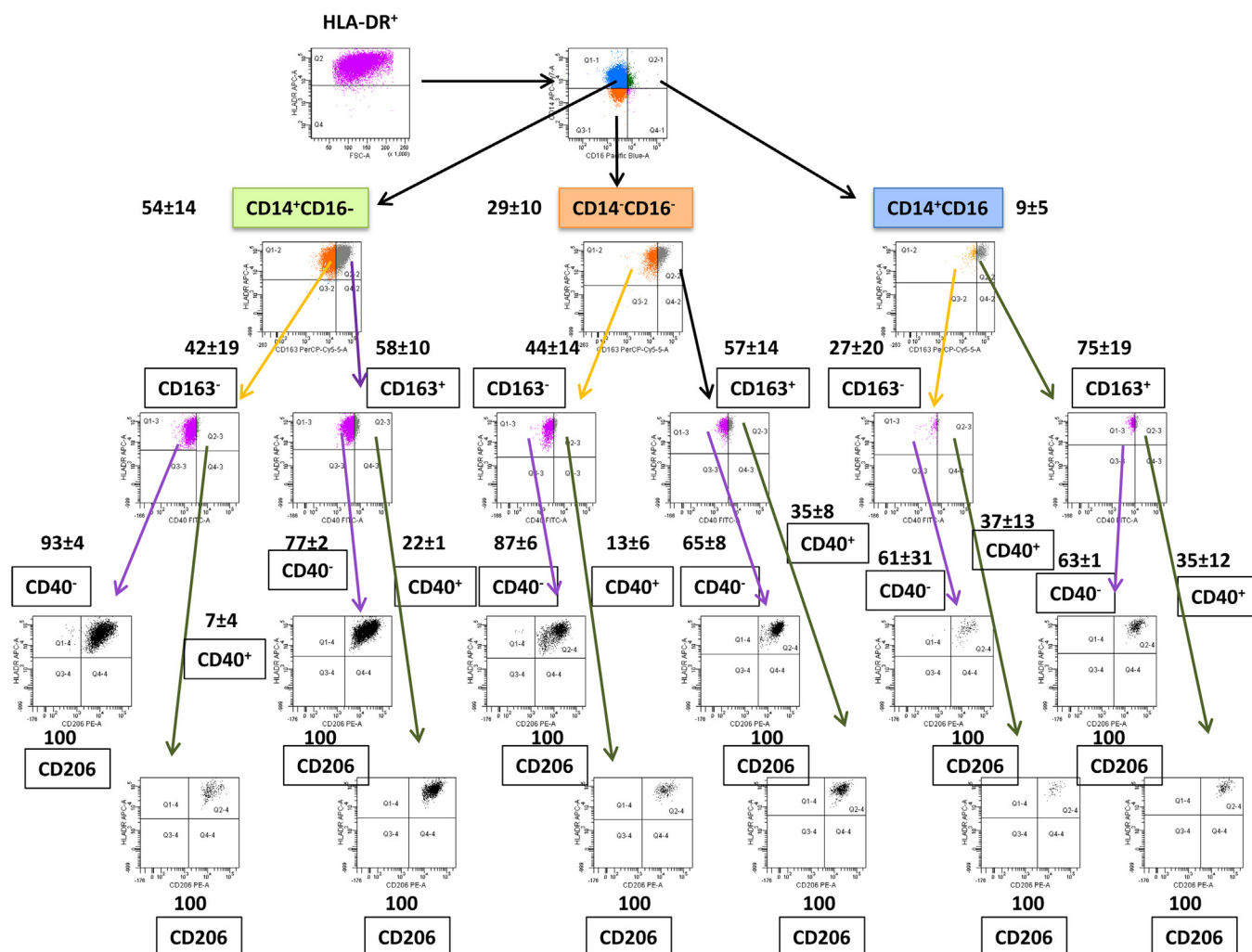
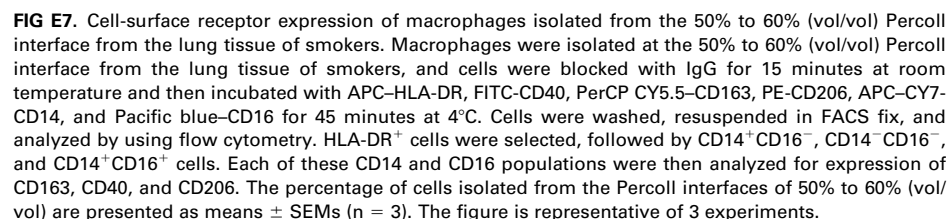


FIG E6. Cell-surface receptor expression of macrophages isolated from the 40% to 50% (vol/vol) Percoll interface from the lung tissue of smokers. Macrophages were isolated at the 40% to 50% (vol/vol) Percoll interface from the lung tissue of smokers, and cells were blocked with IgG for 15 minutes at room temperature and then incubated with APC–HLA-DR, FITC-CD40, PerCP CY5.5–CD163, PE-CD206, APC–CY7-CD14, and Pacific blue–CD16 for 45 minutes at 4°C. Cells were washed, resuspended in FACS fix, and analyzed by using flow cytometry. HLA-DR⁺ cells were selected, followed by CD14⁺CD16⁻, CD14⁻CD16⁺, and CD14⁺CD16⁺ cells. Each of these CD14 and CD16 populations were then analyzed for expression of CD163, CD40, and CD206. The percentage of cells isolated from the Percoll interfaces of 40% to 50% (vol/vol) are presented as means ± SEMs (n = 3). The figure is representative of 3 experiments.



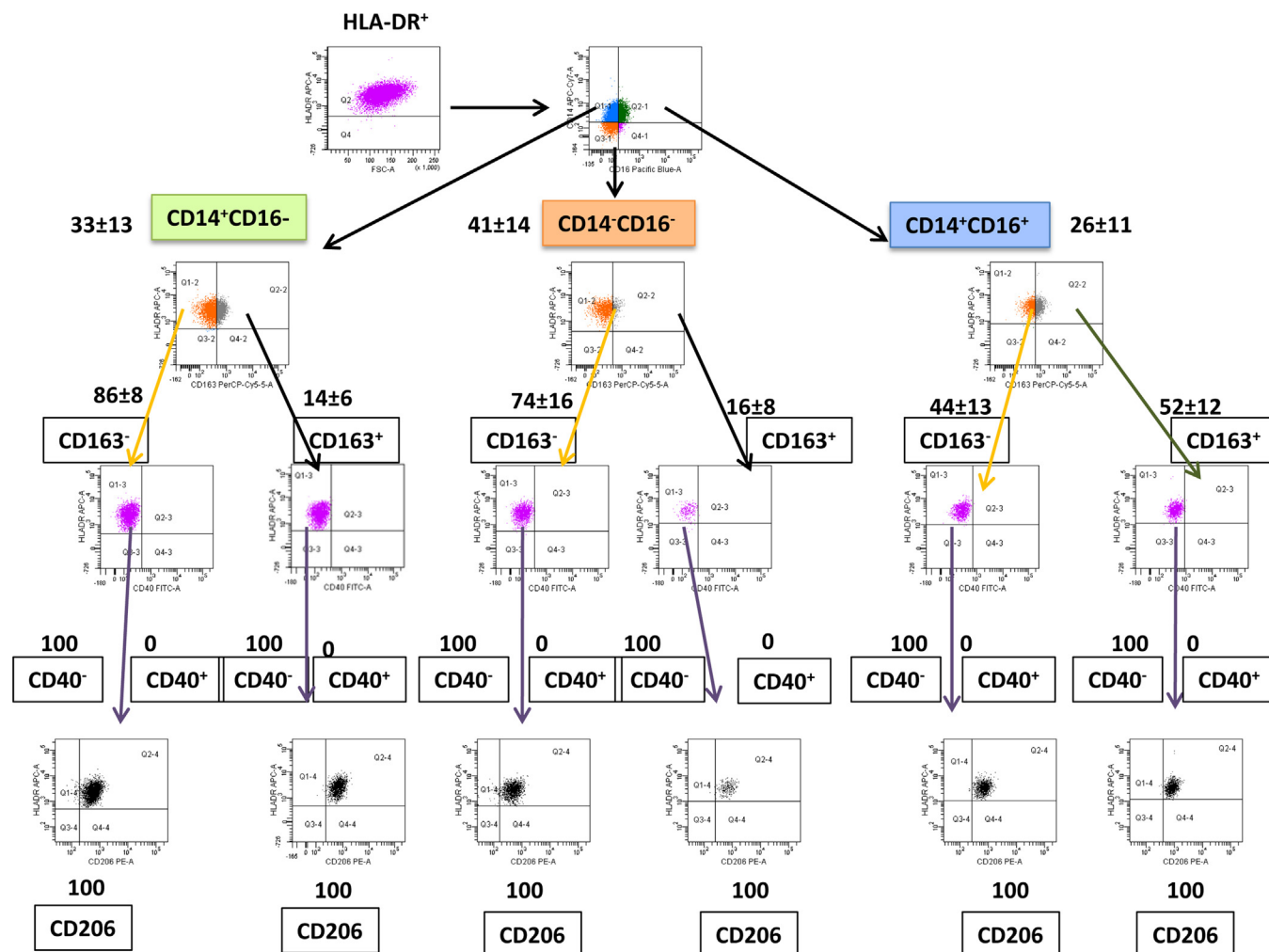


FIG E8. Cell-surface receptor expression of macrophages isolated from the 40% to 50% (vol/vol) Percoll interface from the lung tissue of patients with COPD. Macrophages were isolated at the 40% to 50% (vol/vol) Percoll interface from the lung tissue of patients with COPD, and cells were blocked with IgG for 15 minutes at room temperature and then incubated with APC–HLA-DR, FITC-CD40, PerCP CY5.5–CD163, PE-CD206, APC–CY7–CD14, and Pacific blue–CD16 for 45 minutes at 4°C. Cells were washed, resuspended in FACS fix, and analyzed by using flow cytometry. HLA-DR⁺ cells were selected, followed by CD14⁺CD16⁻, CD14⁻CD16⁻, and CD14⁺CD16⁺ cells. Each of these CD14 and CD16 populations were then analyzed for expression of CD163, CD40, and CD206. The percentage of cells isolated from the Percoll interfaces of 40% to 50% (vol/vol) are presented as means ± SEMs (n = 3). The figure is representative of 3 experiments.

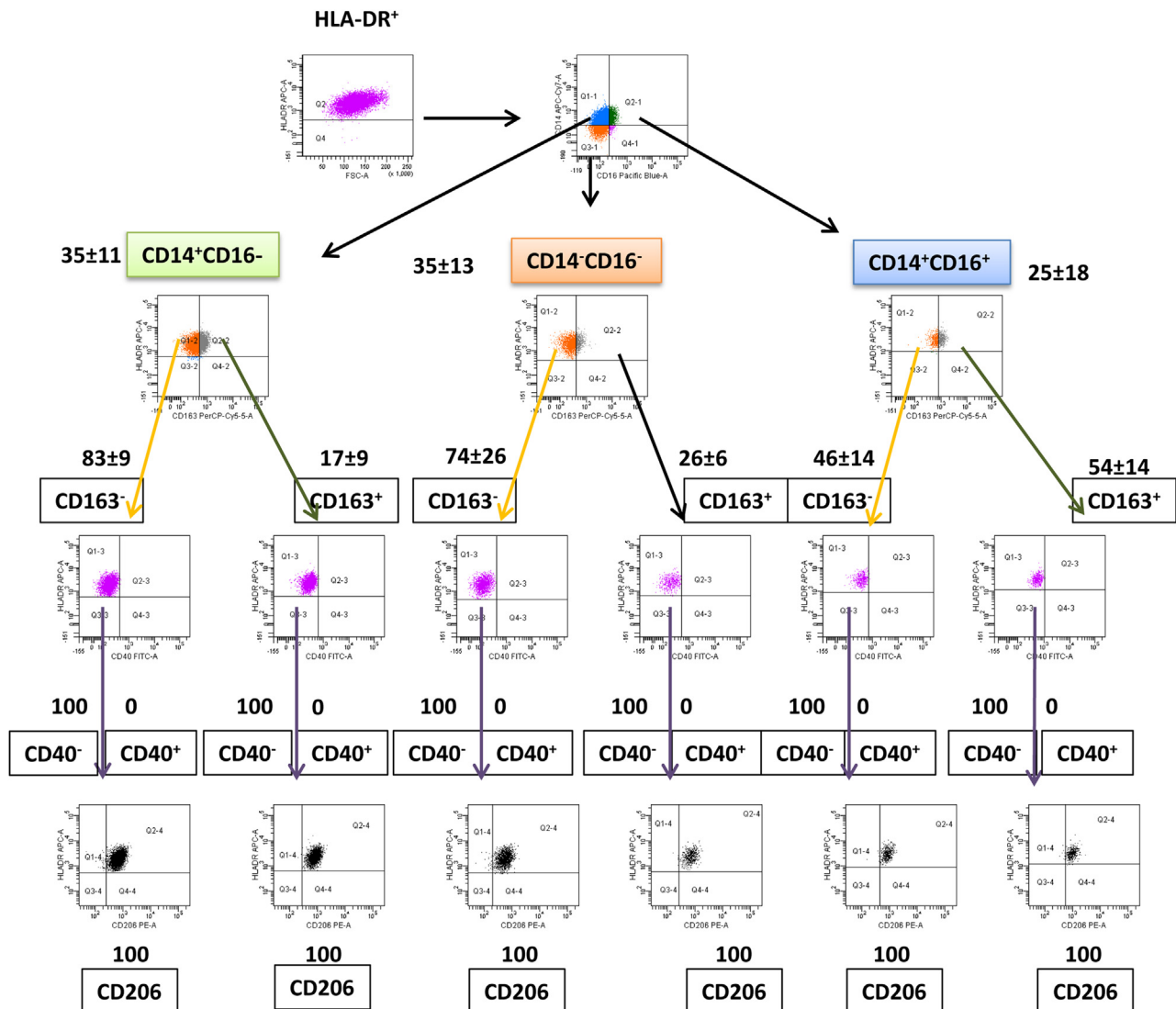


FIG E9. Cell-surface receptor expression of macrophages isolated from the 50% to 60% (vol/vol) Percoll interface from the lung tissue of patients with COPD. Macrophages were isolated at the 50% to 60% (vol/vol) Percoll interface from the lung tissue of smokers, and cells were blocked with IgG for 15 minutes at room temperature and then incubated with APC–HLA-DR, FITC–CD40, PerCP CY5.5–CD163, PE–CD206, APC–CY7–CD14, and Pacific blue–CD16 for 45 minutes at 4°C. Cells were washed, resuspended in FACS fix, and analyzed by using flow cytometry. HLA-DR⁺ cells were selected, followed by CD14⁺CD16⁻, CD14⁺CD16⁺, and CD14⁺CD16⁺ cells. Each of these CD14 and CD16 populations were then analyzed for expression of CD163, CD40, and CD206. The percentage of cells isolated from the Percoll interfaces of 50% to 60% (vol/vol) are presented as means ± SEMs (n = 3). The figure is representative of 3 experiments.

TABLE E1. Distribution of macrophages in each fraction

Percoll fraction	Nonsmokers (n = 4)		Smokers (n = 19)		Patients with COPD (n = 4)	
	M ϕ $\times$ 10 ⁶ /g tissue	Total M ϕ (%)	M ϕ $\times$ 10 ⁶ /g tissue	Total M ϕ (%)	M ϕ $\times$ 10 ⁶ /g tissue	Total M ϕ (%)
30% to 40%	1.9 $\pm$ 0.6	34.9 $\pm$ 4.0	4.7 $\pm$ 0.9	41.2 $\pm$ 3.8	3.3 $\pm$ 1.7	23.9 $\pm$ 3.7
40% to 50%	2.2 $\pm$ 0.8	34.4 $\pm$ 1.7	4.0 $\pm$ 0.6	37.6 $\pm$ 1.4	1.6 $\pm$ 0.4	28.1 $\pm$ 1.6
50% to 60%	0.8 $\pm$ 0.5	33.6 $\pm$ 3.0	2.5 $\pm$ 0.4*	37.2 $\pm$ 1.9	0.8 $\pm$ 0.4	29.2 $\pm$ 2.7

Resected lung tissue was weighed, and macrophages (M ϕ) were isolated from the 30% to 40% (vol/vol), 40% to 50% (vol/vol), and 50% to 60% (vol/vol) interfaces of the Percoll gradient from nonsmokers, smokers, and patients with COPD. Cells were stained with Kimura dye and counted. Data are presented as means $\pm$ SEMs.

* P < .05 versus nonsmokers and patients with COPD.