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PIVOTAL ADVANCE

Pivotal Advance: Expansion of small sputum macrophages in CF: failure to express MARCO and mannose receptors

Adam K. A. Wright, Satish Rao,*,† Simon Range,‡ Christiane Eder,§ Thomas P. J. Hofer,§ Marion Frankenberger,§ Lester Kobzik,|| Chris Brightling,*,‡ Jonathan Grigg,*,¶ and Loems Ziegler-Heitbrock*,§,1*

*Department of Infection, Immunity and Inflammation, University of Leicester, Leicester, United Kingdom; †Department of Respiratory Medicine and Cystic Fibrosis, Birmingham Children's Hospital, Birmingham, United Kingdom; ‡Institute for Lung Health, Glenfield Hospital, University Hospitals of Leicester National Health Service, Leicester, United Kingdom; §Clinical Cooperation Group, Inflammatory Lung Diseases, Asklepios-Fachklinik and Helmholtz Zentrum München, German Research Center for Environmental Health, Gauting, Germany; ||Physiology Program, Harvard School of Public Health, Boston, Massachusetts, USA; and ¶Academic Unit of Paediatrics, Institute of Cell and Molecular Science, Queen Mary University London, London, United Kingdom

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ABSTRACT

Macrophages in the airways form an important element of immune defense and inflammation. We analyzed induced sputum from airways of patients with CF for the types of macrophages present, their receptor expression, and phagocytic function. In samples from patients and age-matched controls, macrophages were analyzed by multicolor flow cytometry, scavenger receptor expression was studied at the protein and mRNA level, and receptor function was investigated using fluorescent particles. In adult patients with CF, we discovered a pronounced expansion of the small CD14+ DR+ CD68weak+ macrophages to 73 ± 18% compared with 16 ± 8% in healthy controls. Expression of the MARCO and CD206 (mannose receptor) was strongly reduced at the mRNA and protein level in sputum macrophages. Antibody-blocking studies showed that MARCO mediates phagocytosis of unopsonized particles. In line with reduced MARCO expression, sputum macrophages in CF showed a deficient uptake of particles (23±9% of cells) compared with healthy controls (71±15%). The deficiency of MARCO expression in the predominant small sputum macrophages in CF may lead to impaired clearance of inhaled particles with increased inflammation and damage to the CF lung. *J. Leukoc. Biol.* 86: 479–489; 2009.

Introduction

CF is caused by a common, autosomal, recessive gene defect. CF is most prevalent in countries with a major Caucasian population such as the United Kingdom (one in 2600) and the United States (one in 3500) [1], and it is a leading genetic cause of mortality in these countries. The gene involved is the CF transmembrane conductance regulator, which is encoded on Chromosome 7 and has, to date, produced over 1600 mutations that are associated with the disease phenotype (http://www.genet.sickkids.on.ca).

These mutations result in a defect in anion transport that disrupts sodium and water transport across cell membranes [2, 3]. In the airways, this leads to a dehydrated composition of airway surface liquid [4] and to production of hyperviscous mucus [5]. Bronchial obstruction then ensues, compromising clearance by the mucociliary system and thereby providing an environment that supports microbial colonization and infection. Frequent infection goes along with pronounced neutrophil accumulation in the airway lumen, and these cells can cause substantial damage over time with eventual respiratory failure [6].

An additional mechanism that may contribute to lung damage in CF calls for an inherent propensity to mount an exaggerated inflammatory response [7]. To this end, stimulation of CF leukocytes has shown increased cytokine production compared with cells from healthy individuals [8–10], and similar findings were obtained in animal models of the disease [11].

Neutrophils are the most prominent cell type in sputum from CF patients [12]. In contrast, in the healthy airways, the macrophage is the predominant phagocyte. Macrophages are important players in processes of immune defense and inflammation as a result of their expression of pattern recognition receptors such as TLRs and scavenger receptors. This confers upon them a wide range of functions such as an ability to per-

Abbreviations: APC=allophycocyanin, CF=cystic fibrosis, COPD=chronic obstructive pulmonary disease, DC-SIGN=dendritic cell-specific ICAM-grabbing nonintegrin, FSC=forward-scatter, MARCO=macrophage receptor with collagenous structure, PC5=phycocyanin 5.5, PLK-1=anti-MARCO antibody, qPCR=quantitative PCR, SSC=side-scatter

1. Correspondence: Helmholtz Zentrum München, German Research Center for Environmental Health, Robert-Koch-Allee 29, 82131 Gauting, Germany. E-mail: zeigler-heitbrock@helmholtz-muenchen.de

form phagocytosis and subsequently destroy bacteria, release potent, proinflammatory cytokines such as TNF, and produce chemokines leading to the attraction of neutrophils. Animal studies have, in fact, shown that macrophages can control immigration of neutrophils [13–15].

Airway macrophages can be sampled via induced sputum, and previously, we have shown that in patients with COPD, a population of small macrophages can predominate [16]. These cells exhibit high levels of CD14 and HLA-DR and produce cytokines and chemokines constitutively, suggesting that they may contribute to inflammation in COPD. In the present study, we therefore have addressed the question of whether in CF, such small macrophages can be detected in the airways and in addition, whether receptors involved in phagocytosis and clearance are intact on CF macrophages. We show herein a pronounced expansion of small macrophages in CF and a strongly reduced expression of scavenger receptors by these cells. These data indicate that clearance of inhaled particles will be impaired in CF airway macrophages, thereby leading to increased damage to the lung.

MATERIALS AND METHODS

Subjects

CF volunteers were seen in the outpatient department of the Royal Infirmary (children) or Glenfield Hospital site (adults) of University Hospitals of Leicester National Health Service Trust (UK). Control volunteers were healthy donors recruited from the university and hospital staff. They were enrolled in the study after written, informed consent had been obtained from the study volunteer or the parent/guardian for volunteers aged 16 or younger. The University Hospitals of Leicester Research Ethics Committee approved volunteer recruitment and the experimental protocol.

Pediatric and adult CF patients with classical disease were identified primarily based on clinical presentation of their disease phenotype, where such patients have presented early with the disease, suffer repeated chest infections, and have other organ (e.g., pancreatic) involvement. In addition, all classical subjects had a positive sweat test (>60 mmol/l), and their diagnosis was confirmed by detection of homo- or heterozygosity for the most frequent CF-causing alleles (**Table 1**).

In contrast, we noted that other adult CF patients with nonclassical CF were diagnosed later in life, had mild lung disease, and required attendance of the out-patient clinic only infrequently, i.e., once or twice/year (Table 1). Their CF diagnosis was confirmed by detection of "mild" CF disease-causing mutations. Sweat-test results for the nonclassical group were not determined for two individuals, borderline in one (57 mmol/l), and positive in another. The CF consultant respiratory physician (S. Range), blinded to the study data, made the distribution of CF study volunteers between classical and nonclassical groups.

Data from COPD patients were obtained using sputum spontaneously produced rather than induced.

Sputum induction and processing

Sputum induction and processing were carried out according to E. Pizzichini and co-workers [17, 18], with some modifications. Briefly, the subjects received 200 μg salbutamol and were asked to inhale a sterile 5.4% hypertonic saline aerosol generated by a multisonic nebulizer set to maximum output for 5 min. Donors were then asked to blow their nose and rinse their mouths with water prior to recording the FEV_1. The participants were encouraged to cough vigorously into a Petri dish. This was repeated for a further two inhalations. In total, saline inhalation for CF and controls was performed for no longer than 15 min, and sputum was processed immediately on ice.

Sputum from CF patients was processed as a whole, and in control donors, plaques were selected to minimize contamination with epithelial cells from the oropharynx as described by Kelly et al. [19]. Donated sputum (1 vol) was then admixed with 4× the volume of 10% 6.5 mM DTT in 100 mM PBS (pH 7.0), reconstituted according to the manufacturer's instructions (Calbiochem, San Diego, CA, USA). The sputum/DTT mixture was placed on ice and rotated on a bench rocker (ThermoScientific, Basingstoke, UK) for 15 min. An equal volume of PBS (pH 7.4) was added subsequently.

The resultant cell suspension was then filtered through a cotton gauze and twice through 48 μm nylon gauze to remove aggregates. Cells were centrifuged at 800 g for 10 min at 4°C. The cell pellet was assessed for cell viability (Table 1) using the trypan blue exclusion method [19]. Cells were pelleted and resuspended in PBS (+2% FCS) for flow cytometry staining and analysis.

TABLE 1. Demographic Data for Adult Control, CF, and COPD Volunteers

	Control	Classical CF	Nonclassical CF	COPD
Donors (*n*)	16	10	4	8
Age (mean years±SD)	35.2 ± 12	21.5 ± 4[a]	42.5 ± 10	73.7 ± 12.6
Sex (female:male)	5:11	7:3	1:3	4:4
Ethnicity (Caucasian: Indian)	12:4	8:2	4:0	8:0
Genotype (*n*)	N/A	ΔF508+/+ (6); ΔF508+/N5098R (1); ΔF508+/unknown (3)	ΔF508+/R117H (2); A455E/A4326delTC (1); G542x/PolyT5/9 (1)	N/A
Last known culture (*n*)	N/A	*Pseudomonas* aeruginosa (9); NSG (1)	NSG (3); *Candida* sp. (1)	*Streptococcus* sp. (2); *Haemophilus* sp. (1); *Pseudomonas* sp. (1); NSG (4)
Inhaled steroids (yes:no)	N/A	5:5	0:4	7:1
% Sputum cell viability (mean±SD)	87 ± 13	78 ± 10[b]	64 ± 16	84 ± 15
% FEV^1 (mean±SD)	89 ± 15	62 ± 19[a]	93 ± 16	38.3 ± 10.3
% Small MΦ (mean±SD)	14.1 ± 8.1	73 ± 18[c]	31 ± 20	83.2 ± 12.3[c]

FEV^1, Forced expiratory volume in 1 s; N/A, not applicable; NSG, no significant growth; MΦ, macrophage; unknown refers to a CF mutation that is outside of those commonly testet. [a]$P = 0.0023$ versus control; [b]$P = 0.03$ versus control; [c]$P < 0.0001$ versus control (unpaired *t*-test); all other comparisons are nonsignificant.

Flow cytometry

Sputum cells were stained with the following specific mouse anti-human mAb or isotype controls according to the manufacturer's recommendations: anti-HLA-DR-PC5 conjugate (IgG_1, A07793, Beckman-Coulter, Krefeld, Germany) and anti-CD14-APC conjugate (IgG_{2a}, #IM2580, Beckman-Coulter). For better differentiation of macrophages from neutrophils, we used anti-CD16b-FITC conjugate (#MCA1725F, Serotec, UK) and anti-CD66b-FITC (#MCA216F, Serotec). To further aid identification of sputum macrophages by intracellular staining, we used CytoFix/CytoPerm kit (#51-2090k2) and Perm/Wash (#51-2091k2) according to the manufacturer's (Becton Dickinson, Wycombe, UK) instructions. Permeabilized sputum cells were then stained with PE-labeled CD68 or IgG_{2b}, (reagent set #556078, Becton Dickinson). PBS was added to increase the volume for cell acquisition on a Becton Dickinson FACSCalibur. The Becton Dickinson FACSCalibur was equipped with a dual-laser (488 nm argon and 633 diode lasers) set-up and photomultiplier tubes for four-color parameter staining. Data analysis was performed using CellQuest Pro software.

Staining for scavenger receptors

To determine expression of cell-surface CD206 (mannose receptor) and CD209 (DC-SIGN), sputum cells were stained with anti-CD16b-FITC, anti-CD66b-FITC, anti-HLA-DR-PerCP (#347402, Becton Dickinson), and anti-CD14-APC as above and anti-CD206-PE (#555954, Becton Dickinson) or CD209-PE (#551265, Becton Dickinson). Nonspecific binding was determined by substituting the specific antibody for the respective IgG_1 and IgG_{2b} isotype control.

For staining of the MARCO, sputum cells were incubated with the primary antibody, anti-human MARCO (clone PLK-1 [20]), at a final concentration of 10 μg/ml (determined by serial dilution) or an isotype control (IgG_3, #M3645, Sigma Chemical Co., St. Louis, MO, USA; 10 μg/ml final) for 30 min on ice. Cells were washed once with PBS (+2% FCS) and resuspended in 100 μl to which 2 μl PE-conjugated secondary antibody goat $F(ab')_2$ anti-mouse (#IM0855, Beckman-Coulter) was added. After 30 min, cells were washed and incubated with 20 μg/ml IgG_3 for 15 min on ice to block nonspecific binding via available binding capacity of the secondary antibody. Sputum cells were then stained by adding anti-CD16b-FITC and anti-CD66b-FITC, anti-CD14-APC, and anti-HLA-DR-PerCP as above.

Purification of sputum macrophages

A sputum cell suspension was obtained as described previously and resuspended in 1 ml PBS (2% FCS). To this cell suspension, mononuclear cell-depleted erythrocytes (30 μl; obtained following centrifugation over Ficoll-Paque) and 50 μl monocyte enrichment cocktail (RosetteSep monocyte enrichment reagent, #15068, Stem Cell Technologies via Cell Systems, St Katharinen, Germany) were added and incubated at room temperature for 20 min. Cells were then diluted 1:1 with tissue-culture grade PBS, layered over an equal volume of Ficoll-Paque density gradient medium (GE Healthcare Uppsala, Sweden), and centrifuged at 800 g for 30 min to generate a mononuclear layer, which was aspirated, washed, and resuspended in serum-free culture medium. Cells were counted, and viability was determined using trypan blue as stated previously.

RT-PCR

qPCR for mRNA was performed as described previously [21]. In brief, lysates containing 2×10^4 cells in 200 μl TRI-Reagent were thawed and spiked with 15 μg tRNA from brewer's yeast (Roche Diagnostics, Mannheim, Germany) as carrier. RNA was isolated according to the manufacture's instructions, and equal amounts of each sample were reverse-transcribed with oligo(dT) as primer. qPCR was performed using the Light Cycler system (Roche Diagnostics) with 3 μl cDNA/capillary in the SYBR Green format using the LightCycler-FastStart DNA Master SYBR Green I kit from Roche Diagnostics (Catalogue #2 239 264). As an external control, the housekeeping gene α-enolase was amplified under the same conditions (annealing at 60°C). Amplification was done with the following primers: MARCO forward, 5′ GTG TCC GTC AGG ATT GTC GG 3′, reverse, 5′ ATA TGA GCC CGA GGA CAC CT 3′; mannose receptor forward, 5′ TAA TTG CTT GTT TTC TAG CC 3′, reverse, 5′ TGG GGG AAA CAA GAT TCT CA 3′. These primers give product sizes of 350 and 399 bp, respectively.

Particle uptake

Purified sputum macrophages (50 μl) were admixed with green fluorescent YG carboxylated polystyrene fluorospheres (1 μm size, #15702, Polysciences, Eppelheim, Germany) at a ratio of 1:5 and samples were incubated for 1 h at 37°C, 5% CO_2. The cells were stained with anti-CD45-APC according to the manufacturer's instructions (#IM 2473, Beckman-Coulter) on ice, and finally, trypan blue at 0.08 μg/ml final was added to quench extracellular fluorescent signals from the intracellular fluorospheres. Samples were analyzed after gating on CD45-positive events and on macrophages based on high FSC and right-angle light-scatter.

For blocking experiments, isolated macrophages were incubated initially on ice with an anti-MARCO antibody (PLK-1) or mouse IgG_3 isotype control (#ab9405, AbCam, Cambridge, UK) at 20 μg/ml final, followed by addition of the fluorospheres.

Statistics

Statistical analysis was carried out using paired and unpaired Student's *t*-test and Mann-Whitney U-test, as described in the text, using GraphPad Prism software (Version 3.0, GraphPad Inc., San Diego, CA, USA). A level of $P < 0.05$ was considered significant.

RESULTS

Identification of small sputum macrophages by flow cytometry

Sputum samples were isolated and stained with mAb against granulocytes and macrophages. As demonstrated in **Figure 1** for a control donor, we first gated on CD14+ HLA-DR+ cells in a CD14-APC versus DR-PC5 dot plot (Fig. 1A). Cells within this gate (R1) were analyzed for expression of CD68-PE versus CD16b-FITC plus CD66b-FITC. This analysis separates contaminating CD16b+CD66b+ granulocytes from CD68+ macrophages (R2 in Fig. 1B). The specificity of the staining is shown for CD14 in Figure 1D and for DR in Figure 1E. Specificity for CD68 is demonstrated in Figure 1F. The light-scatter properties of the CD14+ HLA-DR+ CD68+ cells (Fig. 1C) show that these cells exhibit high size (FSC) and granularity (SSC), indicating that they are large bronchial macrophages. The analysis also demonstrates that CD68 staining is strong and that these cells also have a high green autofluorescence level, a feature known from other types of mature lung macrophages.

Using the same gating strategy, we went on to compare macrophages from a second healthy adult (**Fig. 2, A–C**) with those from an adult CF patient with a classical ΔF508 mutation (Fig. 2, D–F). Sputum cells from the CF patient show that there is again a distinct CD14+ DR+ population (Fig. 2D), and these cells show a low level of expression of CD68 (Fig. 2E) compared with macrophages in a healthy control (Fig. 2B). On average, the CD68 expression in control donor macrophages (n=10) is high at 1013 ± 673 channels, and CD68 was low with 191 ± 170 channels in CF macrophages (n=10; P=0.0003; Mann-Whitney U-test). When looking at the light-scatter characteristics (Fig. 2F), the CD68low cells showed much lower FSC; i.e., they are small macrophages. To support

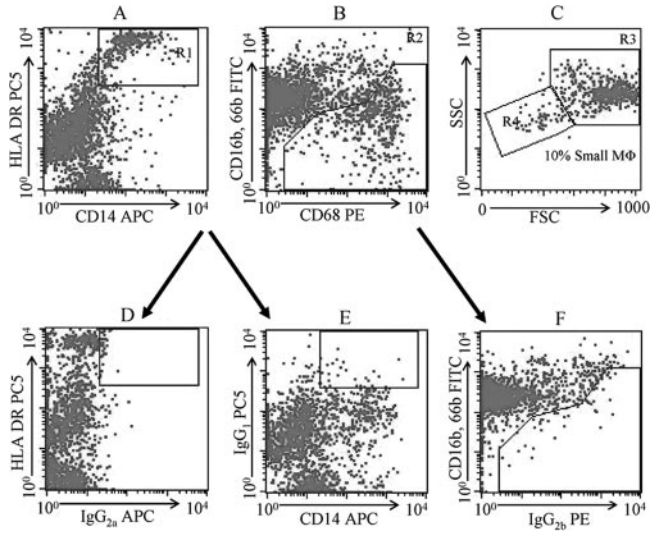

Figure 1. Multicolor flow cytometry staining to identify small sputum macrophages in a healthy adult control donor. Sputum cells from a healthy control donor were stained with anti-CD14-APC, anti HLA-DR-PC5, anti-CD16b-FITC, plus anti-CD66b-FITC and anti-CD68-PE. Positive cells were gated by comparison with the appropriate isotype controls. The CD14+ and DR+ events gated in R1 (A) were analyzed for CD68 expression (B). A gate was set around the CD68+ cells (R2) with exclusion of CD16b+ CD66b+ neutrophils (B). The CD68+ events were then analyzed for light-scatter properties (C). Gate R3 defines large macrophages; gate R4 small macrophages. (D) The isotype control for CD14 (IgG_{2a}) is combined with the specific antibody for DR. (E) The isotype for DR (IgG_1) is combined with the specific antibody for CD14. (F) The isotype control for CD68 (IgG_{2b}) is combined with the anti-granulocyte antibodies. The arrows connect the specific antibody to the respective isotype controls.

this hypothesis, we examined the light-scatter characteristics (FSC and SSC) of sputum macrophages and blood monocytes isolated from a group of five CF donors. Although the minor population of large macrophages had an average signal of 685 ± 19 channels, the small macrophages were significantly lower at 442 ± 24 ($P<0.0001$; Student's *t*-test), and this is similar to blood monocytes at 444 ± 36 channels. When looking at granularity, as reflected by SSC, we found a similar pattern with significantly ($P<0.0001$; Student's *t*-test) lower signals for blood monocytes and the small sputum macrophages and high granule content for the large macrophages (data not shown). These data about low size and granularity in small macrophages are consistent with the concept that these macrophages are immature cells, i.e., newly recruited monocytes from blood.

The percentage of small macrophages in a group of 10 patients with classical CF was at 73 ± 18% (**Fig. 3** and Table 1, bottom line). This included two patients with 39% and 46% small macrophages. These patients did not show any clinical features that would distinguish them from the other classical CF cases in the cohort. The average percentage of the CD14+ HLA-DR+ CD68low small macrophages in the classical CF patients was significantly higher compared with the percentage in the group of 10 healthy controls (16±8%) A group of four cases with nonclassical CF showed intermediate values with an average of 31 ± 20% (not significant; Fig. 3 and Table 1).

Expression of scavenger receptors on sputum macrophages

Macrophages in the lung contribute to immune defense by phagocytosis of inhaled particles and microbes. For this, they use scavenger receptors such as CD206 (mannose receptor), CD209 (DC-SIGN), and MARCO. We have therefore asked whether the small sputum macrophages, which are expanded in CF, would show cell-surface expression of these receptors compared with the predominant large macrophage population in controls.

When analyzing the cell-surface expression of MARCO on control CD14+ HLA-DR+ sputum macrophages (**Fig. 4A**), we noted a strong signal on large macrophages (Fig. 4B; geometric mean, 194) but no expression on the minor population of small macrophages (Fig. 4C; geometric mean, 0). In contrast, analysis of CF macrophages (Fig. 4D) revealed that large mac-

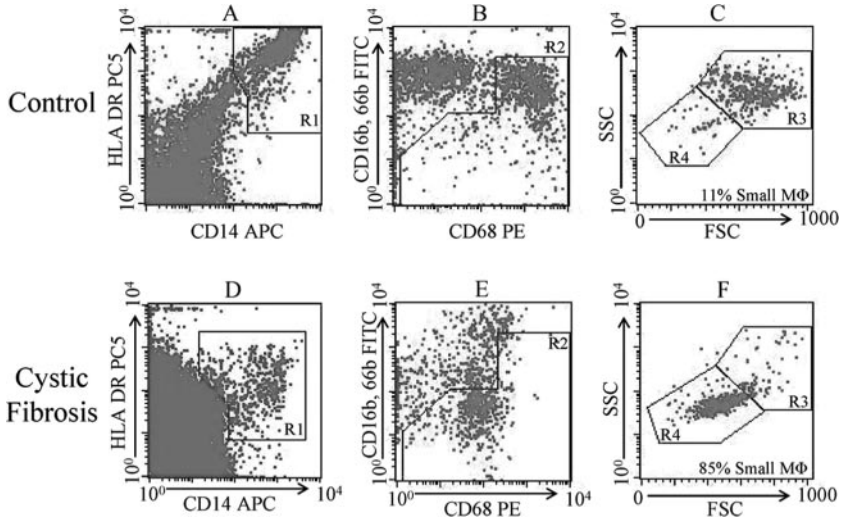

Figure 2. Multicolor flow cytometry analysis of sputum macrophages from an adult CF donor as compared with a control donor. Sputum cells from a healthy control donor (A–C) and from a classical CF patient (D–F) were stained as detailed in the legend to Figure 1. CD14+ and HLA-DR+ events gated in R1 (A and D) were analyzed for CD68 expression (B and E), and the CD68+ cells in R2 (B and E) were analyzed for light-scatter properties (C and F). The percentage of small macrophages in R4 (C and F) was 11% for the control donor and 85% for the classical CF donor.

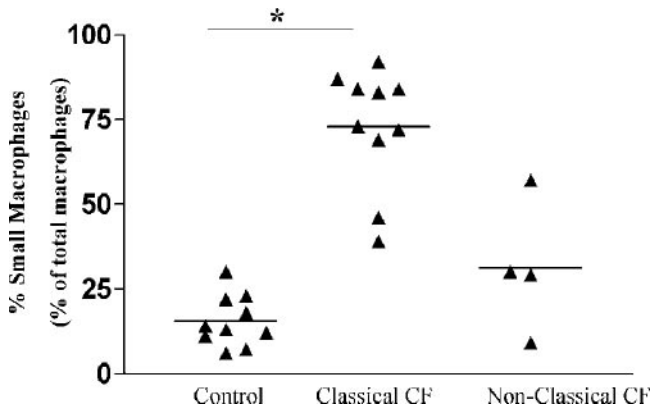

Figure 3. Percentage of small macrophages in adult CF cases and control donors. The percentage of small macrophages in R4 was determined relative to all macrophages given in R3 plus R4. The average value for the controls was 16 ± 8%; for classical CF, it was 73 ± 18%, and for nonclassical CF, it was 31 ± 20%. *, $P < 0.0001$, unpaired *t*-test. Horizontal bars indicate mean values. The age distribution was 35.2 ± 12 years for the healthy control cohort and 21.5 ± 4 years for the classical CF cohort, and for the nonclassical CF cohort, it was 42.5 ± 10 years.

rophages were also positive for MARCO (Fig. 4E; geometric mean, 41), albeit at a lower level compared with the healthy control. Importantly, the small macrophages in CF were negative for MARCO (Fig. 4F).

The analysis of a group of five CF patients and four control donors confirms these findings in that large macrophages from controls were positive for MARCO with a fluorescence intensity of 219 ± 97 channels. In CF, the large macrophages exhibited fluorescence at 33 ± 37 channels, and small macrophages from CF patients and controls did not reveal any MARCO cell-surface expression (**Table 2**). When looking at expression of MARCO for the entire population of macrophages in CF, we found a low average expression level of 9 ± 15 channels compared with 115 ± 77 channels in healthy control donors.

A similar pattern was seen for CD206 (Table 2), where we were able to detect a strong signal on control, large macrophages (963±548) but little or no expression on small macrophages (5±7). Similar to MARCO, we were able to detect low expression on large macrophages from CF donors (16±14), and we were unable to detect CD206 on small macrophages (1±3) from the same donors. When looking at expression of CD206 for the entire population of macrophages in CF, we found a low average expression level of 2 ± 3 channels compared with 542 ± 304 channels in healthy control donors (Table 2).

For CD209, we were unable to detect CD209-specific fluorescence on any sputum macrophage population, except for a weak signal in the few large macrophages in CF (Table 2).

We then asked whether the down-regulation of MARCO and CD206 might be a result of a down-regulation of the respective transcript. For this, we isolated mRNA from purified sputum macrophages and performed RT-PCR. As shown in **Figure 5A**, the amplification curve for MARCO in a control donor showed an increase at a much earlier cycle number compared with a CF patient (Fig. 5B, compare solid lines in Fig. 5, A with B). After correction for different RNA content in the samples by reference to the respective α-enolase, the expres-

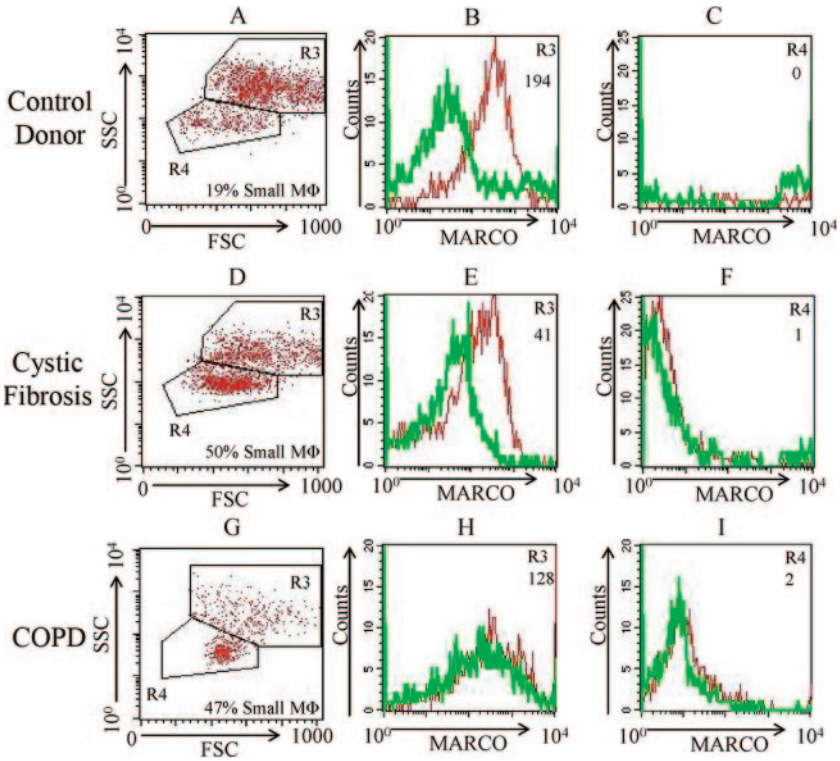

Figure 4. Expression of cell-surface MARCO protein by sputum macrophages from an adult CF donor as compared with a control donor. Sputum samples from a healthy control donor and a classical CF donor were stained with anti-CD14-APC, anti HLA-DR-PerCP, and anti-MARCO or an isotype control. Small and large cells were determined based on light-scatter (A and D), and expression of MARCO on large (R3, B and E) and small (R4, C and F) macrophages was determined. The specific fluorescence intensity (geometric mean channels) for anti-MARCO staining is given in the upper-right corner of the histogram. Red line, Anti-MARCO; green line, isotype control. (G–I) Data from a COPD patient. Of total macrophages, small macrophages accounted for 19% in control samples and 50% in the CF example shown. Similar to CF, small macrophages accounted for 47% of total macrophages in COPD in the example shown.

TABLE 2. Expression of Scavenger Receptors (Geometric Mean Fluorescence Channels±SD) on Sputum Macrophages from Adult Control, CF, and COPD Donors

Receptor expression	Control donors			CF			COPD		
	Large	Small	Total	Large	Small	Total	Large	Small	Total
MARCO	219 ± 97 ($n = 4$)	3 ± 6	115 ± 77	33 ± 37 ($n = 5$)[a]	1 ± 1	9 ± 15[b]	48 ± 69[b] ($n = 3$)	3 ± 1	5 ± 7
CD206	963 ± 548 ($n = 4$)	5 ± 7	542 ± 304	16 ± 14 ($n = 5$)[a]	1 ± 3	2 ± 3[b]	183 ± 190[b] ($n = 4$)	7 ± 4	0 ± 0
CD209	1 ± 2 ($n = 3$)	0 ± 0	1 ± 1	10 ± 11 ($n = 3$)	0 ± 0	1 ± 0	ND	ND	ND
% Macrophages (% of total macrophages)	85 ± 5	12 ± 6	–	35 ± 20	58 ± 18[c]	–	22 ± 21 ($n = 5$)	79 ± 19[c]	–

Values are average geometric mean ± SD. ND, Not determined. [a]$P < 0.01$ versus control; [b]$P < 0.05$ versus control; [c]$P < 0.0001$ versus control (unpaired t-test); all other comparisons are nonsignificant.

sion level for MARCO was 23-fold lower in the CF patient macrophages (Fig. 5B) as compared with the control donor cells in this example (Fig. 5A). The average mRNA levels for the MARCO receptor when comparing transcript levels in CF with levels in control macrophages were 16-fold (compare Columns 2 and 4 in Fig. 5C). For CD206, transcript levels were eightfold lower in CF compared with controls (Columns 1 and 3).

Particle uptake by sputum macrophages

To study scavenger receptor function, we then examined uptake of unopsonized particles by sputum macrophages. In the example from a control donor (**Fig. 6, A–C**), there was a strong increase in green fluorescence by macrophages incubated with 1 μm particles compared with those that were not exposed to particles (Fig. 6, C vs. B). On average, the percentage of control macrophages (n=4) that had taken up particles was 71 ± 15% (**Table 3**). When analyzing particle uptake by large and small macrophages from control donors, this gave an uptake of 75 ± 19% and 33 ± 11%, respectively (Table 3). This demonstrates that in control donors, particle uptake by large macrophages was significantly higher than by small macrophages.

Next, we analyzed whether MARCO, a scavenger receptor, which has been implicated in the uptake of unopsonized particles in murine [22] and human [20, 23] macrophage studies, is also involved in the particle uptake by sputum macrophages. For this, we preincubated isolated cells with anti-MARCO or an isotype antibody followed by culture with particles for 1 h at 37°C. We then analyzed green fluorescence of the particles in the CD45+ macrophages by flow cytometry. These studies gave a mean fluorescence intensity for total macrophages of 125 ± 62 channels in control-treated cells while for anti-MARCO-treated cells, the cell-associated fluorescence for total macrophages was reduced to 72 ± 44 channels (**Fig. 7**). This 42% reduction of particle uptake by the blocking antibody demonstrates that MARCO contributes substantially to the particle uptake by sputum macrophages.

Having shown that MARCO mediates particle uptake, and MARCO receptor expression is reduced in CF, we hypothesized that uptake of particles is reduced in CF macrophages. In fact, there was only a low percentage of cells among all macrophages showing particle uptake (Fig. 6F). On average, the percentage uptake was 23 ± 9% for CF (Table 3, n=3). Also, there was a shift to the left for the geometric mean fluorescence signal in CF samples (Fig. 6F) compared with controls (Fig. 6C). This translates into a reduced mean fluorescence intensity from 378 ± 121 fluorescence channels for controls to 165 ± 15 for CF. These data indicate that the number of particles phagocytosed per macrophage is also reduced in CF.

When looking at percentage uptake by large and small macrophages in CF, both populations showed a reduced uptake with an average 24 ± 13% and 27 ± 9%, respectively (Table 3). Anti-MARCO treatment of CF macrophages could not reduce the low-level phagocytosis in CF macrophages any further. The average fluorescence intensity for isotype-treated and anti-MARCO-treated cells was 7 ± 2 channels (Fig. 7).

These data demonstrate a strongly reduced ability and capacity of CF sputum macrophages to take up particles as a result, in part, of the pronounced down-regulation of the scavenger receptor MARCO.

Sputum macrophages in pediatric CF

When applying the staining protocol for the identification of sputum macrophages to five pediatric CF cases, we found an increased proportion of small macrophages, 50 ± 35%, compared with eight healthy pediatric controls with a lower proportion of 13 ± 13% (**Table 4**). These data demonstrate that in children with CF, a similar increase in the proportion of small macrophages occurs. The paucity of pediatric sputum material precluded a more detailed analysis of receptors and function.

Sputum macrophages in COPD

COPD and CF show a similar clinical presentation with cough and sputum production, recurrent airway infection leading to remodeling, and destruction of the lung. We therefore analyzed sputum macrophages from eight COPD donors (Table

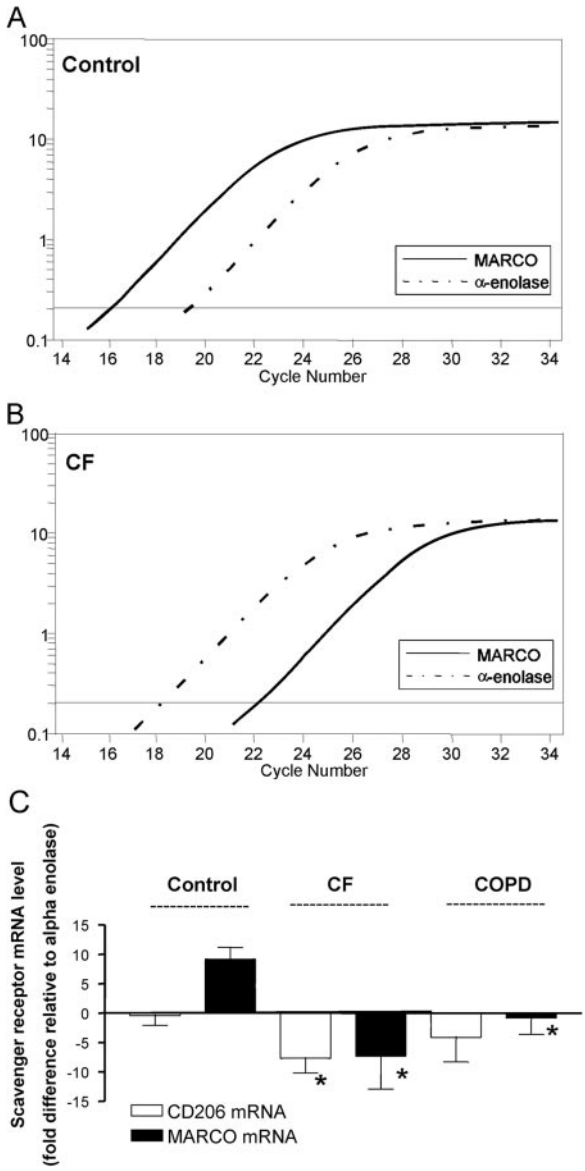

Figure 5. Expression of MARCO mRNA by sputum macrophages in adult CF and COPD samples compared with healthy controls. Macrophages were separated from sputum and RT-PCR for MARCO, and CD206 was performed relative to the expression of α-enolase. (A and B) Representative examples of light cycler data showing cDNA amplification of MARCO (solid line) and the housekeeping gene α-enolase (dashed line) from control and CF donor macrophages, respectively. Note that the amplification curves for MARCO start to rise before α-enolase in controls (A) but after α-enolase in CF macrophages (B). (C) The abundance of CD206 (open bars) and MARCO (solid bars) mRNA isolated from control, CF, and COPD macrophages following correction for α-enolase content. Controls, $n = 3$; CF, $n = 3$; COPD, $n = 3$; *, $P < 0.01$, compared with controls using an unpaired *t*-test.

1) for cellular composition and receptor expression. In line with our earlier findings, COPD patients showed a strong increase in the proportion of small macrophages to an average of 83 ± 12% (Table 1). Similar to adult CF, there was also a pronounced down-regulation of cell-surface MARCO protein expression (Fig. 4, G–I). MARCO expression was low on large and small sputum macrophages, with an average fluorescence of 48 ± 69 and 3 ± 1 channels, respectively (Table 2). CD206 cell-surface protein was also low on COPD large and small macrophages, with an average of 183 ± 190 and 7 ± 4 channels, respectively (Table 2). In line with the reduced scavenger receptor protein, purified macrophages from an additional set of three COPD donors showed a significant tenfold reduction of mRNA for MARCO, which was tenfold lower compared with control macrophages (Fig. 5C, compare Columns 2 and 6). CD206 mRNA was also reduced, but this was less pronounced when compared with the reduction seen in CF (not significant). Hence, COPD sputum macrophages show a comparable increase in small macrophages and similar down-regulation of scavenger receptors at the mRNA and protein level as seen in CF.

DISCUSSION

CF has long been considered a pediatric disease, but progress in therapy, including regular use of antibiotics, glucocorticoids, and mucolytics, has led to patients surviving well into adulthood. The pathology in CF is determined to a large extent by infection and inflammation, and there is evidence that inflammation may be an early event preceding infection in the disease [24]. The chronic, recurrent infection and inflammation lead to lung damage and eventually to organ failure.

Among the inflammatory cells that are present in the airway lumen, the neutrophilic granulocytes predominate, and they apparently can cause much of the damage to the lung. On the other hand, macrophages with their ability to produce cytokines and chemokines can orchestrate inflammation and can regulate recruitment of neutrophils to the lung [13, 15]. Although the importance of macrophages has been recognized in the related lung disease COPD [25–27], this is not the case for CF.

We have looked at macrophages in adults with classical CF, and for this, we have developed a flow cytometry approach to identify these cells in sputum samples. This six-parameter approach uses different mAb to exclude granulocytes and to identify and characterize macrophages positively in CF. With this technique, we were able to show for the first time that CF sputa contain increased numbers of macrophages with low size and low expression levels of the macrophage marker CD68. These features are in line with the conclusion that these cells are less mature than the large macrophages, as with maturation, macrophages typically increase in size and in expression of CD68 [28]. Such small macrophages have been detected in the alveolar space in patients with interstitial lung disease [29], in acute respiratory distress syndrome [30], and in sputum samples of COPD patients [16]. For CF, this is the first report to show an increase of these cells. As to the origin of these small macrophages, the most likely explanation is that these cells are monocytes, newly migrated from blood into the lung in a chemokine-dependent manner.

CCL2 is a chemokine that can drive recruitment of monocytes into the lung [13, 31]. Levels of CCL2 were found increased in COPD sputum [32], and we can demonstrate increased levels of

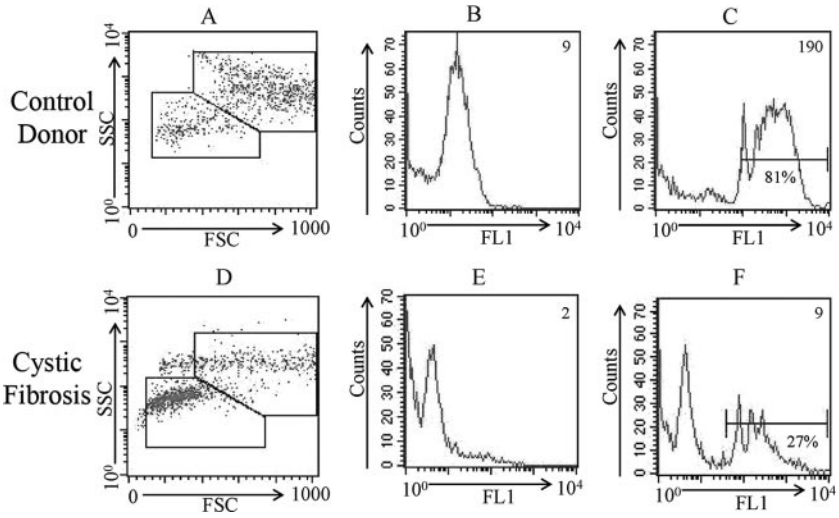

Figure 6. Phagocytosis by sputum macrophages in adult CF compared with a healthy control. Control (A–C) and CF (D–F) macrophages were separated from sputum samples and incubated for 1 h with latex particles followed by flow cytometry. Given is the scatter profile (A and D) and the fluorescence of total macrophages incubated without (B and E) or with (C and F) particles. The geometric mean fluorescence is given in the upper-right corner of each histogram. (C and F) Bars indicate the percentage of cells with cell-associated fluorescence, and the discrete peaks of fluorescence result from distinct numbers of particles/cell. Here, the peak of lowest intensity represents one particle/cell. Data are representative of four controls and three CF cases, as summarized in Figure 7 and Table 3. FL1, Fluorescence 1.

this chemokine also in CF [33]. Hence, CCL2 may be responsible for the increase of small macrophages in the disease.

Nonclassical CF patients, who have milder disease [34], also had only a mild increase in small macrophages in sputum. This indicates that numbers of small bronchial macrophages are related to the degree of inflammation and damage. It suggests that these cells may be crucial towards inflammation in the lung. However, more cases with nonclassical CF need to be studied to substantiate this point. Further to this, the pediatric cases with classical CF in our study showed less of an increase of small macrophages compared with the adult, classical CF cases in line with a less-pronounced degree of inflammation in the early stages of CF.

Macrophages in the airways form a first line of defense against inhaled particles and microbes, and they do so by phagocytosis, which uses cell-surface receptors that are involved in binding and internalization of particles and microbes. We have studied such receptors and have examined two C-type lectin receptors: the mannose receptor (CD206) and DC-SIGN (CD209) and the scavenger receptor MARCO. Although DC-SIGN was not expressed on sputum macrophages at all, we found strong cell-surface expression for CD206 and MARCO when looking at macrophages from healthy individuals. Expression was restricted to large macrophages, and the few small macrophages were essentially negative. On blood monocytes, cell-surface MARCO and CD206 were absent, supporting the concept that the small sputum macrophages in healthy individuals may derive directly from blood monocytes.

In the present study, we found that expression of these receptors in CF was decreased substantially on the large macrophages, and it was negative on small macrophages. As the latter predominate in CF sputum, the overall expression is low for CD206 and MARCO. This down-regulation may be a result of protease-mediated removal of the cell-surface receptor protein—a mechanism that has been demonstrated to account for decreased $CXCR_1$ expression on CF neutrophils [35] and for the reduction of cell-surface receptors on alveolar macrophages in CF [36]. On the other hand, the low levels can be a result of down-regulation of the respective mRNA. In fact, we can demonstrate a substantially decreased level for CD206 and MARCO transcripts in CF sputum macrophages compared with control (Fig. 5). This indicates that proteolysis is not crucially involved in the decrease of these receptors in CF macrophages.

In this study, we could confirm earlier work [16] about the expansion of small macrophages in sputum samples from COPD patients. When looking at these samples for expression of CD206 and MARCO at mRNA and cell-surface protein level, we found a similar decrease to that seen in CF. The decreased expression of CD206 in COPD has been noted recently for alveolar macrophages [37], but in that study, no data are provided about small macrophages in the samples studied.

Our findings described here and elsewhere [16] suggest that there are similar pathophysiological processes, occurring in CF and COPD, which could account for reduced expression of scavenger receptors. Indeed proinflammatory cytokines such as TNF are elevated in the CF and COPD airways [12, 38–40]; also, TNF and IFN-γ have been demonstrated to reduce substantially expression of CD206 mRNA and protein [41, 42] and MARCO protein [43] on rodent macrophages. Further-

TABLE 3. Particle Uptake (Mean Fluorescence Channels±SD) by Sputum Macrophages from Adult Control and CF Donors

	Control donors (n = 4)			CF (n = 3)		
	Large	Small	Total	Large	Small	Total
Phagocytosis (%)	75 ± 19	33 ± 11[a]	71 ± 15	24 ± 13	27 ± 9	23 ± 9[b]

[a] $P < 0.01$ versus control large macrophages; [b] $P < 0.01$ versus control total macrophages using an unpaired Student's *t-test*.

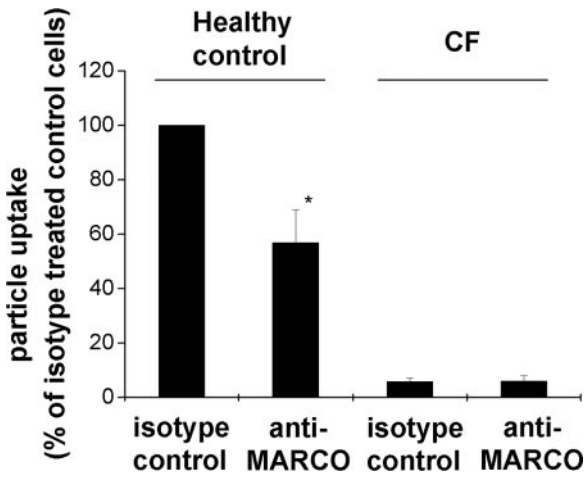

Figure 7. Blockade of particle phagocytosis by anti-MARCO antibody. Purified sputum macrophages were incubated with an anti-MARCO antibody or isotype control, followed by incubation with latex particles for 1 h. Particle uptake was determined by recording cell-associated fluorescence (geometric mean intensity). To compare the different treatment groups easily, the geometric mean fluorescence intensity for anti-MARCO-treated control and CF macrophages is expressed as percentage of isotype-treated controls; $n = 4$ for controls, and $n = 3$ for CF. *, $P < 0.05$, compared with isotype control using a paired *t*-test.

more, the transcript levels for CD206 and MARCO may be low in the patients, as the majority of the macrophages has immature cells, newly immigrated from blood, and the blood monocytes are essentially negative for these receptors.

The role of the CD206 mannose receptor in immune defense is not completely clear. Although the receptor can bind opportunistic pathogens such as *Candida albicans*, *Pneumocystis carinii*, and *Streptococcus pneumoniae* [44], for CD206−/− mice, increased susceptibility to infection has not been detected [45, 46]. This indicates that CD206 does not play a crucial role in antimicrobial defense. Rather, the CD206 molecule may be involved in clearance of endogenous glycoproteins, which increase during inflammation [47].

The MARCO receptor can bind Gram-negative microbial organisms such as *P. aeruginosa* via LPS [48] and Gram-positive organisms *Staphylococcus aureus* and *S. pneumoniae* via currently unknown bacterial structures [20, 22]. Knockout mice do show an immunodeficiency phenotype with increased bacteria numbers and lower survival upon experimental infection with *S. pneumoniae* [22]; however, pneumococcal infection does not appear to be a major problem in CF. On the other hand, *P. aeruginosa* (as shown in this manuscript) and *S. aureus* are the most prominent infectious organisms isolated from CF patients [34], and CF sputum contains high quantities of endotoxin (LPS) [49]. Because of the ability of MARCO to interact with these bacteria, we suggest that the increased susceptibility to infection with these organisms in CF may be, in part, a result of the pronounced MARCO deficiency demonstrated herein. Further, MARCO has been demonstrated to be important for clearance of oxidized lipids, such that inhalation of ozone and subsequent generation of lipids such as β-epoxide will lead to enhanced inflammation [50]. In addition, MARCO can bind and mediate internalization of unopsonized particles including nano-particles [20]. Clearance of such particles may be another important function of MARCO.

We have studied uptake of unopsonized particles by sputum macrophages, and by antibody-blocking studies, we can confirm that MARCO is crucial to uptake (Fig. 7). Anti-MARCO treatment could not reduce uptake completely. This may be explained by an insufficient avidity of the antibody or by an insufficient antibody concentration. The concentration used herein (20 μg/ml) was in the range that had been used previously [20], but we cannot exclude that a higher concentration would have been more effective in our system. Another explanation for the incomplete blockade is that other receptors may be involved in the particle uptake.

In line with reduced expression of MARCO on CF macrophages, we found a strongly decreased particle uptake by CF macrophages. These data suggest that the increase of small macrophages and the concomitant decrease of scavenger receptors such as MARCO in CF may lead to an inability to

TABLE 4. Demographic Data for Pediatric Control and CF Volunteers

	Control	CF
Donors (*n*)	8	5
Age (mean years±SD)	12.8 ± 3	12.2 ± 3
Sex (female:male)	4:4	3:2
Ethnicity (Caucasian:Indian)	6:2	4:1
Genotype (*n*)	N/A	ΔF508+/+(3); ΔF508+/R347 (1); ΔF508+/G551D (1)
Last known culture (*n*)	N/A	NSG (2); *Mycobacterium* sp. (1); *P. aeruginosa* (1); *Aspergillus* sp. (1)
Inhaled steroids (yes:no)	N/A	2:3
% Sputum cell viability (mean±SD)	67 ± 9	63 ± 15
% FEV[1] (mean±SD)	96 ± 10	80 ± 20
% Small MΦ (mean±SD)	13 ± 13	50 ± 35[a]

[a] $P = 0.0193$ versus control (unpaired *t*-test); all other comparisons are nonsignificant.

properly clear inhaled particles such as air pollutants and microbes.

Taken together, the absence of scavenger receptors such as CD206 and MARCO on airway macrophages may result in a higher susceptibility to infection, and based on the failure to clear inflammatory glycoproteins, oxidized lipids, and inhaled particles, this may contribute to an enhanced inflammation and damage in the CF airways.

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cystic fibrosis · phagocytosis · scavenger receptor