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Smoking-dependent Reprogramming of Alveolar Macrophage Polarization: Implication for Pathogenesis of COPD*

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Abstract

When exposed to specific microenvironment, macrophages acquire either M1- or M2-polarized phenotypes associated with inflammation and tissue remodeling, respectively. Alveolar macrophages (AM) directly interact with environmental stimuli such as cigarette smoke, the major risk factor for chronic obstructive pulmonary disease (COPD), a disease characterized by lung inflammation and remodeling. Transcriptional profiling of AM obtained by bronchoalveolar lavage of 24 healthy nonsmokers, 34 healthy smokers and 12 COPD smokers was performed to test the hypothesis whether smoking alters AM polarization resulting in a disease-relevant activation phenotype. The analysis revealed that AM of healthy smokers exhibited a unique polarization pattern characterized by substantial suppression of M1-related inflammatory/immune genes and induction of genes associated with various M2-polarization programs relevant to tissue remodeling and immunoregulation. Such reciprocal changes progressed with the development of COPD with M1-related gene expression being most dramatically down-regulated ($p<0.0001$ vs healthy nonsmokers, $p<0.002$ vs healthy smokers), results confirmed with TaqMan real-time PCR and flow cytometry. Among progressively down-regulated M1-related genes were those encoding type I chemokines CXCL9, CXCL10, CXCL11, and CCL5. Progressive activation of M2-related program was characterized by induction of tissue remodeling and immunoregulatory genes such as MMP2, MMP7 and ADORA3. Principal component analysis revealed that differential expression of polarization-related genes has substantial contribution to global AM phenotypes associated with smoking and COPD. In summary, the data provides transcriptome-based evidence that AM likely contribute to COPD pathogenesis in non-inflammatory manner due to their smoking-induced reprogramming towards M1-deactivated, partially M2-polarized macrophages.

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Introduction

Mononuclear phagocytes are heterogeneous population of cells with significant phenotypic plasticity (1). Depending on the microenvironment, they undergo distinct activation programs acquiring polarized phenotypes and different functional capacities that together provide an armamentarium that helps to protect, repair and sometimes damage tissues (2–4). Mononuclear phagocyte "M1 polarization", also referred to as the "classical activation" program, is induced by signals generated during Th1-mediated immune response such as interferon (IFN) γ and by exposure to pathogen components such as bacterial lipopolysaccharide (LPS) (2–4). The M1 polarization response is characterized by up-regulation of genes relevant to inflammation and cell-mediated immunity. In contrast, mononuclear phagocyte "M2 polarization", induced upon exposure to the Th2 cytokines IL-4 and IL-13 (referred to as "alternative activation") or immunoregulatory signals such as IL-10 (also called "deactivation") and glucocorticoids, is highlighted by induction of expression of receptors with scavenger functions, anti-inflammatory cytokines and molecules implicated in tissue remodeling (1–4).

Although considerable evidence has accumulated regarding the reprogramming of human blood monocytes and murine macrophages *in vitro* depending on the environment to which they are exposed, little attention has been paid in defining how the *in vivo* environment modifies the global polarization program of human macrophages in health and disease. Alveolar macrophages (AM), the pulmonary representatives of the mononuclear phagocyte system, play a central role in defending the lung against pathogens and other environmental challenges, as well as in mediating damage and repair in the lung parenchyma (5,6). AM are unique among mononuclear phagocytes in that AM mostly reside on the respiratory epithelial surface, and thus are exposed directly to the outside environment. One of the most common of these environmental exposures is cigarette smoking, the major risk factor for the development of chronic obstructive pulmonary disease (COPD) (7) that is currently a leading cause of morbidity and mortality worldwide (7,8). Studies in experimental animals and humans have led to the concept that AM play a central role in the pathogenesis of COPD as a major source of mediators that derange the normal lung structure (6,9). In humans, AM numbers are increased in the lung of healthy smokers and individuals with COPD, AM accumulate in areas of lung destruction, and there is a correlation between the AM numbers, airway obstruction and severity of COPD (6,9–12).

Based on studies in murine transgenic models and several human studies that suggest that IFNγ-dependent inflammation is responsible for the development of smoking-induced lung disease (10,13–16), and reports indicating up-regulation of genes related to scavenger function, anti-inflammatory cytokines and remodeling mediators in AM of smokers (17,18), we hypothesized that, compared to healthy nonsmokers, AM of healthy smokers demonstrate an altered polarization program, and that this polarization pattern progresses with the development of COPD. To assess this hypothesis, global transcriptional profiles were used to assess the M1 and M2 polarization-related genes in AM of 24 healthy nonsmokers, 34 healthy and 12 COPD smokers using Affymetrix microarrays with TaqMan real-time PCR and FACS confirmation of the phenotypic changes. The data demonstrates that cigarette smoke does indeed alter the steady-state polarization status of human AM *in vivo*, including induction of several genes representing M2 sub-phenotypes. Surprisingly, however, rather than up-regulating the M1 polarization program as expected, cigarette smoking induces in AM of healthy smokers the opposite phenotype, characterized by a substantial down-regulation of the majority of the genes associated with M1 polarization. The overall expression of M1-related genes was progressively further down-regulated in COPD smokers accompanied with a gradual up-regulation of some M2-related genes, suggesting that the transcriptome of AM in COPD smokers is characterized by progressive reciprocal dysregulation of M1- and M2-polarization patterns. The data supports the concept that AM contribute to tissue remodeling during the development of

smoking-induced lung disease. However, the data also suggests that it is unlikely that AM play a significant role as inflammatory cells in the early pathogenesis of smoking-induced COPD, a departure from the concept that AM-mediated inflammation participates in the early derangements of the lung induced by smoking.

Methods

Study Population

A total of 70 subjects were assessed including, healthy never-smokers with normal lung function (n=24, referred to as “healthy nonsmokers”), healthy smokers with normal lung function (n=34, “healthy smokers”), and COPD smokers (GOLD classification, n=12, Table I). No COPD smokers with current exacerbation were included in the study. The study was approved by the Weill Cornell Medical College Institutional Review Board and written informed consent was obtained from each individual before enrollment in the study. Subjects were evaluated at the Weill Cornell NIH General Clinical Research Center and Department of Genetic Medicine Clinical Research Facility based on clinical history, physical examination, routine blood screening tests, urinalysis, chest X-ray, ECG and pulmonary function tests. Current smoking status was confirmed by history, venous carboxyhemoglobin levels, and urinalysis for nicotine levels and its derivative cotinine.

Alveolar Macrophages

AM were collected by bronchoalveolar lavage as previously described (19). The total volume used per site was typically 100 ml and a maximum of 3 sites were lavaged per individual. Recovery of the infused volume ranged from 45 to 65%. BAL fluid was filtered with gauze and centrifuged at 1,200 rpm for 5 min, at 4°C. Cells were washed twice in RPMI 1640 containing 10% fetal bovine serum, 50 U/ml penicillin, 50 U/ml streptomycin and 2 mM glutamine (Invitrogen, Carlsbad, CA), suspended in 10 ml medium. Cell viability was assessed by trypan blue exclusion and expressed as a percentage of the total cells recovered. Total cell number was determined by counting on a hemocytometer. Differential cell count was performed on sedimented cells prepared by cytocentrifugation (Cytospin 3; Shandon Instruments, Pittsburgh, PA) stained with DiffQuik reagents (Dade Behring, Newark, NJ) and performed by counting 500 cells on each slide. For the microarray analysis, the remaining cells were seeded in six-well plastic culture dishes (2×10^6 per 2 ml/well) and the AM purified by adherence at 37°C, 2 hr in a 5% CO_2 humidified incubator, removing any nonadherent cells by washing with RPMI 1640 before RNA extraction. Light microscopy was used to assess the morphological features of the cells. After the adherence step, all samples were >98% AM. For FACS analysis, the cells were processed immediately after isolation as described below.

cDNA Preparation and Affymetrix Microarrays

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) followed by RNeasy (Qiagen, Valencia, CA) to remove residual DNA, yielding 2 to 4 μg RNA per 10^6 cells. RNA samples were stored in RNA Secure (Ambion, Austin, TX) at −80°C. RNA integrity was determined by running an aliquot of each RNA sample on an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). A NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE) was used to determine the concentration of RNA. Double stranded cDNA was synthesized from 3 μg of total RNA using the GeneChip One-Cycle cDNA Synthesis Kit, followed by cleanup with GeneChip Sample Cleanup Module, *in vitro* transcription reaction using the GeneChip IVT Labeling Kit, and cleanup and quantification of the biotin-labeled cRNA yield by spectrophotometric analysis (all kits from Affymetrix, Santa Clara, CA). Hybridizations to test chips and to the HG-U133 Plus 2.0 array (54,000 probe sets representing approximately 47,000 full-length human transcripts) were performed according to Affymetrix protocols, processed by the Affymetrix GeneChip Fluidics Station

450 and scanned with an Affymetrix GeneChip Scanner 2500. Overall microarray quality was verified by the following criteria: (1) RNA Integrity Number (RIN) >=7.0; (2) 3'/5' ratio for GAPDH <=3; (3) scaling factor <=10.0; and (4) expression level for all 100 housekeeping genes (as defined by Affymetrix, www.affymetrix.com) with coefficient of variation of <40%. The captured image data from the HG-U133 Plus 2.0 arrays was processed using MAS5 algorithm (Affymetrix Microarray Suite Version 5 software). MAS5-processed data was normalized using GeneSpring version 7.3.1 (Agilent technologies) by setting measurements <0.01 to 0.01, per array, by dividing the raw data by the 50th percentile of all measurements, and per gene, by dividing the raw data by the median expression level for all the genes across all arrays in a data set.

Genes that were significantly modified between 2 groups were identified according to the following criteria: (1) P call of "Present" in ≥20% of samples; (2) magnitude of fold change in average expression value for 2 comparative groups ≥1.5; and (3) $p<0.05$ between the groups with a Benjamini-Hochberg correction to limit the false positive rate (20). To exclude the effect of adherence step of AM purification on global transcriptional changes associated with smoking, we compared our results with the signature of significant smoking-responsive genes identified in a previous study in which AM were isolated by flow cytometry (17).

Characterization of M1 and M2-related Gene Expression

The genes representing the M1 and M2 polarization patterns were chosen based on the literature regarding *in vitro* studies of human and murine monocytes and macrophages (2–4,21–32). A gene was classified as "M1-related" based on the current definition of M1 polarization (classical activation) of mononuclear phagocytes as a molecular pattern that may be induced in macrophages and/or monocytes upon stimulation with IFNγ and / or LPS (2–4,23,24,26, 29,30). A gene was classified as "M2-related" if it can be induced either by Th2 cytokines IL-4 and IL-13 (alternative activation) (2–4,27,29), IL-10 (deactivation) (22,25,28), transforming growth factor-beta (TGF-β) (21,22,32), glucocorticoids (31), or macrophage colony stimulating factor (M-CSF) (29). A gene was also classified as M2 if it has been observed to be down-regulated upon exposure to M1-polarization stimuli (33). Genes that can be induced during both M1 and M2 macrophage polarization (for example, HLA-related genes (3)) were excluded from the analysis. Based on these criteria, 41 M1-related (Table II) and 33 M2-related (Table III) genes were selected for analysis.

The M1 and M2 polarization patterns were determined by comparing, for each probe, the significance of the fold-change in the level of expression among groups. If more than 1 probe from the same gene was on the array, the gene was considered significantly up- or down-regulated in one group compared to another if ≥50% of the probes were significantly changed and/or the change was confirmed by TaqMan analysis.

The polarization pattern of AM associated with cigarette smoking was determined based on the analysis of expression of M1- and M2-related genes in AM of healthy smokers as compared to healthy nonsmokers. M1- and/or M2 genes were determined to be smoking responsive if their expression changed significantly in healthy smokers compared to healthy nonsmokers.

The polarization pattern of AM of COPD smokers was assessed by comparing the expression of M1- and M2-related genes of COPD smokers with that of healthy nonsmokers and healthy smokers. The global transcriptional changes related to M1- and M2-polarization of healthy smokers compared to healthy nonsmokers as well as COPD smokers compared to both healthy nonsmokers and healthy smokers were analyzed by assessing the mean normalized expression levels of all M1-related and M2-related probe sets for healthy nonsmokers, healthy smokers and COPD smokers, using all (i.e., significantly changed as well as those not significantly changed) M1- and M2-related probe sets with P calls ≥20%. To assess whether changes in M1-

and M2-gene expression in AM of COPD smokers were related to smoking, an additional comparative analysis was performed using the M1- and M2-related probe sets significantly differently expressed between COPD smokers and healthy nonsmokers.

Unsupervised hierarchical clustering of the healthy nonsmokers *vs* healthy smokers was carried out for the significantly changed M1- and M2-related genes using the MAS5-analyzed data with the Spearman correlation as similarity measure and the complete linkage clustering algorithm using GeneSpring software. To visualize the differences in expression of the M1- and M2-related genes among the 3 groups (healthy nonsmokers, healthy smokers, COPD-smokers), principal component analysis (PCA) was performed using all M1- and M2-related probe sets with P calls >20% for each group. For comparison, all probe sets on the array were independently assessed with the identical individuals. These analyses were carried out using GeneSpring by mean centering of microarray normalized intensity values of all subjects or the average for each of the three groups in order to assign the general variability in the data to a reduced set of principal components (34). The first 3 principal components containing most of the variance-based information were visualized in 3-dimensional space. To evaluate the relative contribution of the differences in M1- and M2-gene expression to the global transcriptional differences between the study groups, the variability (as measured by the first 3 principal components) determined by PCA of M1- and M2-related probe sets was compared to that revealed by PCA of the same study groups using all 26,959 probe sets with a P call ≥20%. To exclude the possible influence of differences in age between COPD smokers and other groups as well as differences in pack-yr between COPD smokers and healthy smokers, nonparametric Spearman correlation analysis has been used to evaluate the correlation of COPD-relevant M1- and M2-related gene expression with age in healthy smokers and smokers with COPD as well as with pack-yr in smokers with COPD.

Real-time PCR Confirmation of Microarray Data

TaqMan real-time RT-PCR was used to confirm differential expression of a subset of genes found to be differentially expressed by microarray, using the same RNA samples that were used for the microarray analysis. cDNA was synthesized from 2 μg RNA isolated from AM in a 100 μl reaction volume, using the TaqMan Reverse Transciptase Reaction Kit (Applied Biosystems, Foster City, CA) with random hexamers as primers. Two dilutions of 1:10 and 1:100 were made from each sample and duplicate wells were run with each dilution. TaqMan PCR reactions were carried out using pre-made kits from Applied Biosystems and 2 μl of cDNA were used in each 25 μl reaction volume. The PCR reactions were run in an Applied Biosystems Sequence Detection System 7500 and relative expression levels were determined using the $\Delta\Delta C_t$ method using β-actin as endogenous control. TaqMan gene expression assays (all from Applied Biosystems) were assessed for selected M1-related genes [*CD80* (Hs00175478_m1), *IL1B* (Hs00174097_s1), *TNFSF10* (Hs00234356_m1), *CXCL9* (Hs00171065_m1), *CXCL11* (Hs00171138_m1), *GBP5* (Hs00369472_m1), *CCL5* (Hs00174575_m1)], and selected M2-related genes [*CCR5* (Hs99999149_s1), *CD36* (Hs01567186_m1), *ADORA3* (Hs00252933_m1), *MMP2* (Hs00165949_m1), and *MMP7* (Hs01042795_m1)].

Flow Cytometry

To assess AM expression of M1 and M2-related genes at the protein level, freshly isolated cells recovered by lavage (~2×10^6) were washed in PBS containing 2% bovine serum albumin (BSA), resuspended in PBS, 2% BSA and 5% inactivated human serum for 20 min, at 4°C to block nonspecific antibody binding. The cells were stained for surface antigens with the following mouse monoclonal anti-human antibodies: fluorescein (FITC)-conjugated antibodies against CD206 (macrophage mannose receptor, to verify the macrophage phenotype), CD3 (to identify contaminating T cells), phycoerythrin (PE)-conjugated

antibodies against CD56 (to identify contaminating NK cells), CD15 (to identify contaminating granulocytes), TNFSF10 and CD80 (for validation of M1 polarization); and CCR5 and CD36 (for validation of M2 polarization; all from BD Biosciences). Appropriate isotype controls were used for each antibody. The incubation with the antibodies was for 30 min on ice in dark according to the manufacturer's instructions. After subsequent washing, cells were fixed and permeabilized with Cytofix/Cytoperm reagent (BD Biosciences) for 20 min on ice and washed with Perm/Wash solution. For intracellular staining, cells were fixed and permeabilized with Cytofix/Cytoperm reagent (BD Biosciences) for 20 min, 4°C, and washed twice with Perm/Wash solution prior to incubation with the following mouse monoclonal anti-human antibodies: FITC-conjugated antibodies against CCL5 or CXCL9, and PE-conjugated IL1B (all from R&D Systems). All samples were prepared and analyzed with and without quenching with 0.2% crystal violet (1 min, 4°C; Polyscientific, Bayshore, NY) to reduce the autofluorescence (35). After washing, cells were analyzed by a FACScalibur cytometer (BD Biosciences, Pharmingen) using Cell Quest software. Thirty thousand events were collected for each sample. FACS data were analyzed using WinMDI 2.8 software (The Scripps Institute, La Jolla, CA) and expressed as a percentage increase of mean fluorescence for a given antibody over isotype control.

Statistical Analysis

Statistical comparisons for microarray data were calculated using GeneSpring software and associated two-tailed t test with unequal variance. Differences with a fold-change >1.5 and $p<0.05$ with a Benjamini-Hochberg correction were considered statistically significant. For other experiments, comparisons between groups were analyzed by 2-tailed t test, or ANOVA for experiments with more than two subgroups, and values are displayed as mean ± standard deviation.

Deposition of Data

All gene expression data has been deposited at the Gene Expression Omnibus (GEO) site (http://www.ncbi.nlm.nih.gov/geo/). Accession number is as follows: HG-U133 Plus 2.0 GSE13896.

Results

Biologic Samples

The total numbers of cells recovered by lavage for healthy nonsmokers was $12.7 \pm 8.1 \times 10^6$, healthy smokers $25.5 \pm 17.0 \times 10^6$ ($p<0.05$ compared to healthy nonsmokers) and COPD smokers $12.9 \pm 8.1 \times 10^6$ ($p<0.05$ compared to healthy nonsmokers, $p>0.05$ compared to healthy smokers). After purification, the AM populations were >98% pure in all groups. The cell viability ranged from 94 to 97%. To exclude the effect of adherence step of AM purification on global transcriptional changes associated with smoking, we compared the expression of smoking-responsive genes identified in a previous study in which AM were isolated by flow cytometry (17) with our results. Such analysis is relevant for interpretation of data since it has been shown that macrophage may change expression of a set of genes following *in vitro* culture (36). The data shows that the method of AM purification does not significantly modify the differential expression of smoking-responsive genes: around 46% gene probe sets found to be significantly up- or down-regulated in smokers in a study with flow cytometry-based AM isolation had similar significant change in our study (Supplemental Figure 1A) and there is significant positive correlation of smoking-responsive probe set expression detected in these two studies ($r=0.71$, $p<0.0001$; Supplemental Figure 1B).

Deactivation of the Steady-state M1 Polarization Program in AM of Healthy Smokers

Since an altered inflammatory response of AM to cigarette smoking is thought to play a central role in the development of pathological changes leading to chronic lung disease (9,10) and IFNγ, an inducer of M1 macrophage polarization, has been identified as important mediator of smoking-induced disease in animal models (13–15), we first asked whether chronic cigarette smoking skews human AM activation toward M1 polarization *in vivo*? To evaluate this question, the expression of the set of M1-related genes by AM of healthy smokers was compared to that of healthy nonsmokers. Surprisingly, the data showed that the majority of genes associated with M1 polarization were down-regulated in AM of healthy smokers as compared to healthy nonsmokers (Figure 1A, Table II). Of the 41 M1 genes evaluated, 23 genes (56%) were down-regulated in healthy smokers.

Many of the M1-related genes down-regulated in AM by smoking encode T-cell-recruiting chemokines. Among these were IFNγ-inducible type 1 chemokines C-X-C chemokine ligand (CXCL) 11 (the most down-regulated gene, fold-change 7.65 *vs* healthy nonsmokers; $p<0.01$), CXCL9 ($p<0.02$), C-C chemokine ligand (CCL)5 ($p<0.01$), CCL4 ($p<0.02$), as well as a family of proteins implicated in IFN signaling such as guanylate binding proteins (GBP) 1, 2, 4, 5. Among other down-regulated M1-related genes were IFNγ-dependent gene CD69 ($p<0.0003$), costimulatory molecule CD80 ($p<0.002$), inflammasome-related cytokines IL-1 ($p<0.02$) and IL-18 ($p<0.0006$), TNF-related proteins TNFAIP6 ($p<0.01$) and TNFSF10 ($p<0.02$), as well as complement-related genes such as complement factor B and complement component 3a receptor. The TLR4, a LPS receptor, was the only M1-related gene up-regulated in AM of healthy smokers (fold-change 1.5, $p<0.05$). Interestingly, the IL-12 gene, typical for M1 polarization (2–4), was very weakly expressed in AM, there was a tendency (albeit not significant) for its down-regulation in AM of healthy smokers. Another typical M1-related gene, inducible nitric oxide synthase, was not expressed in AM regardless of smoking status, consistent with previous observations in human macrophages (37).

Interestingly, the decreased average expression of many M1-related genes was associated with a marked increase in the number of subjects in the group of healthy smokers in which these genes were not detected at all. For example, only 27% of healthy smokers expressed CD69 as compared to 83% of healthy nonsmokers ($p<10^{-6}$); less than half of healthy smokers (47%) expressed CD80 gene *vs* 96% of healthy nonsmokers ($p<10^{-6}$; Table II). A dramatic smoking-related decrease of P calls was also detected for several chemokine genes including CXCL11 (18% in healthy smokers *vs* 63% in healthy nonsmokers; $p<0.001$) and CXCL10 (47% in healthy smokers *vs* 83% in healthy nonsmokers; $p<0.005$).

The differential expression of several M1-related genes between healthy smokers and healthy nonsmokers was further confirmed by TaqMan real-time PCR (Supplemental Figure 2). For example, the M1-related genes CD80, TNFSF10, IL1B, CXCL11, CXCL9, and CCL5 were all observed to be down-regulated by both microarray and TaqMan PCR. Consistent with these observations, M1-related gene expression was also observed to be down-regulated at the protein level (Supplemental Figure 3). FACS analysis demonstrated that CD80, TNFSF10, IL1B, CXCL9, and CCL5 are down-regulated in AM of healthy smokers compared to healthy nonsmokers AM ($p<0.05$, all comparisons).

Unusual M2-like Polarization Program Induced in AM of Healthy Smokers

The current concept of macrophage polarization programs suggests their reciprocal regulation, implying that M2 polarization develops when IFNγ, the inducer of macrophage activation towards M1-polarization, does not dominate over alternative group of stimuli such as IL-4, IL-13, IL-10, TGFβ, glucocorticoids, and M-CSF and others (3,4). We sought, therefore, to determine whether down-regulation of M1-related gene expression pattern in human AM by

chronic cigarette smoking is accompanied by induction of M2 polarization program?. To assess this question, the expression of M2-related genes was analyzed in AM of healthy smokers in comparison to healthy nonsmokers. Interestingly, by contrast to M1-related genes, the expression of all of the genes associated with M2-like polarization that were significantly modified by smoking were up-regulated in healthy smokers (Table II, Table III, Figure 1B).

However, unlike the broad and distinct pattern of M2-polarization observed with some stimuli, smoking was not associated with a uniform induction of genes related to particular M2 polarization pathway. For example, the genes known to be induced during alternative, IL-4 driven, macrophage activation *in vitro* such as those encoding mannose receptor, macrophage scavenger receptor 1, DECTIN1, and CD163 (2,3) were not significantly modulated by smoking (Table III). Further, expression of other M2-typical genes, such as arginase-1 and YM1 (not shown), were not detected in AM of either group, consistent with previous studies of human macrophages (38). Overall, the panel of smoking-induced M2-related probe sets in AM represented a mixture of different subtypes of M2-like macrophage polarization with simultaneous up-regulation of genes related to IL-4-induced (CD36; $p<0.02$), glucocorticoid-induced (MERTK; $p<0.006$), IL-10-induced (C-C chemokine receptor (CCR)5; $p<0.02$), M-CSF-induced (MMP2; $p<0.03$, and CD36; $p<0.02$), as well as IFNγ-down-regulated (CD9; $p<0.01$) M2-like polarization programs (Table III).

The expression of several M2-related genes was analyzed using TaqMan real-time PCR (Supplemental Figure 2). As examples, MMP2, CCR5 and CD36 were up-regulated in both the microarray and TaqMan analysis, confirming the microarray data regarding their differential expression in AM of healthy smokers *vs* healthy nonsmokers. Furthermore, the M2 genes up-regulated at the mRNA level were also up-regulated at the protein level. For example, FACS analysis showed that CCR5 and CD36 were found to be significantly up-regulated at the protein level in AM of healthy smokers *vs* healthy nonsmokers ($p<0.05$ and $p<0.01$, respectively; Supplemental Figure 3).

Biologic Segregation of Healthy Smokers and Healthy Nonsmokers Based on Expression of M1- and M2-related Genes in AM

Based on the observation of reciprocal dysregulation of M1 and M2 polarization programs in human AM by cigarette smoking, we asked whether the expression of M1- and M2-related genes may serve as a biologic discriminator between healthy smokers and healthy nonsmokers and what is the inter-individual variability of M1- and M2- gene expression among these groups? To address this issue, the normalized expression data of all probe sets for M1- and M2-related genes was analyzed at the personalized level, i.e., for every healthy smoker and healthy nonsmoker assessed in the study. There was a coordinate down-regulation of M1-related gene expression in the majority, but not all healthy smokers, similar to coordinate relatively high expression levels of the same probe sets in the majority, but not all healthy nonsmokers (Figure 2A). The difference between healthy smokers and healthy nonsmokers based on the comparative quantitative evaluation of the average normalized expression levels of all M1-related probe sets in both groups was highly significant ($p<10^{-15}$; Figure 3A). In contrast, the personalized M2-related gene expression profile demonstrated a tendency towards higher overall expression in healthy smokers (Figure 2B). The difference in the overall expression of all M2-related probe sets between the healthy nonsmokers and healthy smokers was significant, albeit less so than for the M1 gene ($p<0.001$; Figure 3C).

To analyze this in a greater detail, we performed hierarchical clustering of healthy smokers and healthy nonsmokers using Spearman correlation analysis and significantly regulated M1- and M2-related gene probe sets as input data set (Figure 2C). This analysis revealed that healthy smokers as a group could be quite effectively segregated from healthy nonsmokers based on expression of smoking-sensitive M1- and M2-related gene probe sets in AM, with only 1 of

24 healthy non-smokers similar to healthy smokers. Remarkable heterogeneity was observed among healthy smokers based on the expression of genes related to macrophage polarization. Indeed, 24 of 34 healthy smokers (71%) exhibited an expression pattern strictly different from healthy nonsmokers, characterized by almost coordinate reciprocal alteration of M1- and M2-related gene expression (Figure 2C). Interestingly, the remaining (29%) of healthy smokers were clustered together with healthy nonsmokers. Together, this suggests that the expression profile of M1- and M2-related genes in AM not only discriminates among healthy smokers and healthy nonsmokers, but also identifies different biologic phenotypes within each group.

Progressive Deactivation of M1-Gene Expression Program in AM with the Development of Smoking-induced Lung Disease

Based on these observations of alteration in M1- and M2-related polarization changes in AM of healthy smokers *vs* healthy nonsmokers, we assessed whether transcriptional changes induced in AM of healthy individuals by chronic cigarette smoking progresses with the development of smoking-induced lung disease. First, we asked whether there is a shift in AM polarization from M1-deactivated to M1-polarized phenotype when smokers develop COPD, a disease hypothesized to be dependent on cigarette smoking-induced IFNγ-mediated inflammatory response in the lung (10,13–16,39). To answer this question, expression of all M1-related gene probe sets in AM of COPD smokers was compared to that of healthy smokers and healthy nonsmokers. In the COPD smokers, the overall average expression of M1-related gene probe sets was substantially suppressed as compared to healthy nonsmokers ($p<10^{-17}$; Figure 3A). Further, COPD smokers exhibited a further down-regulated M1-related gene expression pattern, significantly suppressed compared to healthy smokers ($p<0.002$). The down-regulation of M1-related genes appeared to be rather smoking-dependent than disease-dependent, i.e., there were more M1-related genes that were significantly down-regulated in healthy smokers *vs* healthy nonsmokers than COPD smokers *vs* healthy smokers (Table II). Notably, all M1-related genes significantly down-regulated in COPD smokers *vs* healthy nonsmokers were suppressed to some extend in healthy smokers as compared to nonsmokers. Thus, the earliest changes in the M1 polarization pattern relevant to COPD are already present in smokers without disease, with a progression of deactivation of M1-related gene expression with the development of smoking-induced lung disease. Among the genes progressively down-regulated with the development of smoking-induced lung disease were the M1-related IFNγ-inducible genes CXCL11, CXCL9, and GBP5 (Figure 4A), a phenotypic pattern that was confirmed by TaqMan real-time PCR (Supplemental Figure 4A). The similar trend was characteristic for other M1-related genes including CXCL10, IL1B, and IRF7 (Table II). Consistent with the concept of progressive suppression of expression of the M1-related genes with the development of disease, many M1-related genes were not detected in AM of a considerable portion of COPD smokers. For example, CXCL10 was expressed in AM of only 2 of 12 (17%) COPD smokers whereas it was detected in 92% of healthy nonsmokers and 50% of healthy smokers (Table II). Similar progressive decrease of P calls was noted for other M1-related genes including CXCL11, CD69, CAMP, GBP4, GBP5 (Table II).

Since COPD smokers are significantly older than healthy nonsmokers and smokers (Table I), it is essential to confirm that observed changes in M1-related gene expression are due to smoking but not the older age. If expression of COPD-relevant M1-related genes decreases with age independently on smoking, such tendency should be seen in a general population, such as healthy non-smokers. To exclude this, we analyzed correlation between the expression of COPD-relevant M1-probesets and the age in healthy nonsmokers using Spearman correlation analysis. We found that 9 of 30 (30%) M1-related probe sets have significant positive correlation with age (Supplemental Figure 5A), an opposite direction to expected if assume that down-regulation of M1-related genes in COPD smokers is due to their older age. Only one M1-related gene (TNFAIP6) had a significant negative correlation with age in healthy

nonsmokers, however it did not correlate significantly with age in COPD smokers (Supplemental Figure 5B). Only one out of 30 COPD-relevant M1-related probe sets (IRG1) significantly negatively correlated with age (Supplemental Figure 5B). In addition, we excluded that progressive down-regulation of M1-related probe sets in COPD smokers is not due to longer pack-years as compared to healthy smokers (Table II). Only one M1-related probe set (SOCS3) significantly negatively correlated with pack-years in smokers with COPD (Supplemental Figure 7A). Thus, global down-regulated expression of M1-related genes in smokers with COPD is unlikely due to their older age or longer smoking experience.

Progressive Induction of M2-related Gene Expression Program in AM with the Development of Smoking-induced Lung Disease

We then asked whether M2-like transcriptional pattern of AM observed in healthy smokers progresses with the development of COPD in parallel with a progressive deactivation of M1 polarization program? To answer this question, expression of all M2-related probe sets was analyzed in all three groups. Reciprocal to the observations with the M1-related genes, the overall expression of M2-related gene probe sets was progressively increased with the development of COPD (Figure 3C; $p<0.002$ *vs* healthy nonsmokers). Similar to alterations in M1-related gene expression, the up-regulation of M2-related probe sets in AM of COPD smokers is likely initiated by smoking and not disease itself, since the earliest changes in expression of M2-related probe sets relevant to COPD, albeit not significant, were already observed in smokers without disease (Figure 3D; Table III). Among progressively up-regulated M2-related genes were those encoding adenosine A3 receptor (ADORA3), matrix metalloproteases MMP2 and MMP7 (Figure 4B; Table III), whose expression pattern was confirmed by TaqMan real-time PCR (Supplemental Figure 4B). Interestingly, CCL23, one of markers of IL-4-driven alternative macrophage activation (29,40), was the only significantly down-regulated M2-related gene in AM of COPD smokers (Table III), suggesting that the M2-like phenotype of AM induced by smoking is complex, and differs from that described for alternatively activated macrophages based on the *in vitro* studies.

The possible effect of older age and longer pack-yr on M2-related changes in AM of COPD smokers was excluded similarly as for M1-related genes. Only 1 of 13 COPD-relevant M2-related probe sets (MMP7) has a significant negative correlation with age in healthy nonsmokers (Supplemental Figure 6A), an opposite direction to expected if assume that induction of M2-related probe sets in COPD smokers is due to general increase of their expression with ageing. In the COPD group, none of M2-related COPD-relevant probe sets significantly correlated with age (Supplemental Figure 6B). Only one M2-related probe set (MMP2) significantly correlated with pack-yr in smokers with COPD (Supplemental Figure 7B, respectively). Interestingly, COPD smokers with longer pack-years had significantly lower MMP2 expression (Supplemental Figure 7B), what is opposite to expectation based on assumption that increased expression of M2-related genes in COPD smokers is due to their higher pack-year values. Thus, global up-regulated expression of M2-related genes similarly to down-regulated expression of M1-related genes in smokers with COPD is unlikely due to their older age or longer smoking experience.

Overall Differences in M1- and M2-Related Gene Expression in AM of Healthy Smokers and COPD Smokers

To determine how changes in expression of both M1- and M2-related genes collectively contribute to differences between healthy nonsmokers, healthy smokers and COPD smokers, principal component analysis was first applied to all 26,959 probe sets with P call≥20% compared to the experimental data set of all 114 M1- and M2-related gene probe sets. In the global 26,959-probe set space, subjects of different groups were not clearly separated from each other, indicating heterogeneity within the study groups based on the global transcriptome

patterns, with only 31% of variation in the system represented by the first three principal components (Figure 5A, left panel). However, when the analysis was limited to the expression of M1- and M2-polarization related genes in AM, the first three principal components in this 114- probe set space captured 57% variation of gene expression among the subjects (Figure 5B, left panel). In the 3-dimensional space generated based on expression of M1- and M2-related probe sets, healthy nonsmokers and COPD smokers formed two separate clusters. Consistent with results of hierarchical clustering, principal component analysis on polarization-related genes revealed a substantial heterogeneity of healthy smokers, with some clustering with healthy nonsmokers, while others were similar to COPD smokers (Figure 5B, left panel).

Next, the averaged expression patterns for each group were subjected to principal component analysis. When analyzed as groups, healthy nonsmokers, healthy smokers and smokers with COPD were clearly segregated from each other in the global 26,959-probe set principal component space with a 67.7% of variation detected by the first component (Figure 5A, right panel), suggesting that the global average AM transcriptomes in these three groups are indeed quite different. However, when the averaged representatives for each group were plotted in the 3-dimensional principal component space based on the expression of all M1- and M2-related probe sets, the variation among the groups captured by the first component increased to 86.7% (Figure 5B, right panel). Based on visualization of principal components, gene expression of the AM of COPD smokers were clearly different from AM of healthy nonsmokers, with healthy smokers being between these two groups, consistent with the data showing that smoking-associated reprogramming of AM polarization progresses with the development of COPD.

Discussion

In this study, we employed the concept of macrophage polarization to help understand the role of AM in smoking-induced lung disease. The complex pathogenesis of COPD, a human disease associated with cigarette smoking, is clearly environment-dependent, similar to polarization phenotypes that are acquired by mononuclear phagocytes depending on particular environmental settings. The two major macrophage activation programs, referred to as M1- and M2-polarization, define the ability of macrophages to play a role in inflammation and regulation of tissue integrity, respectively (2–4). Both of these macrophage functions are altered in COPD, a disease in which the remodeling of the airways and lung parenchyma is accompanied by, and thought to be dependent on, an abnormal inflammatory response-induced by cigarette smoking (7,9–12,39,41–43). In the present study, we applied a global transcriptional profiling to assess the polarization pattern of AM in healthy smokers and COPD smokers as compared to healthy nonsmokers. The data demonstrates that cigarette smoking, indeed, alters the steady-state polarization program in human AM *in vivo*. However, contrary to the widespread concept that non-related inflammation is a central driver in the early pathogenesis of COPD, smoking was associated with a substantial down-regulation of genes related to M1 macrophage polarization, i.e., there is an overall hypoinflammatory gene expression pattern in AM of healthy smokers. Deactivation of M1 polarization pattern was accompanied by the induction of an unusual phenotype characterized by up-regulation of genes associated with different M2 polarization programs. The data further demonstrates that, with the development of COPD, there is progression of suppression of the M1 program and, to a lesser extent, enhanced expression of some M2-related genes.

Deactivation of M1 Polarization Program in AM by Cigarette Smoking

A major observation of the present study is that AM of healthy smokers exhibit a coordinate down-regulation of a considerable number of genes typical for M1 polarization, a distinct activation program of inflammatory and host defense genes induced in mononuclear phagocytes *in vitro* by IFNγ and LPS (2–4). Among these genes are those encoding type 1

chemokines CXCL11, CXCL9, CCL4, CCL5, inflammasome-related cytokines IL-1β and IL-18, costimulatory molecule CD80, complement-related proteins, a number of proteins involved in IFNγ signaling. This observation leads to several conclusions.

First, the smoking-induced suppression of the M1 polarization program of AM, and of the inflammation program of AM in general, has implications for understanding the role of AM as inflammatory cells in the pathogenesis of the early events in smoking-induced lung disease. The current concepts of COPD suggest that the disease develops as a result of abnormal inflammatory response of the lung to cigarette smoke or other noxious gases and particles, and this inflammatory response mediates small airways derangements and alveolar destruction (7,9–12,42,43). The lungs of smokers contain increased numbers of AM, which accumulate in the sites of alveolar wall destruction (6,9–12). While there is clear evidence that inflammatory and immune mechanisms play a role in mediating lung damage in established COPD (7,9,10, 16,41–43), the effect of smoking on the pro-inflammatory properties of individual cell types in the human lung *in vivo* before the manifestation of the disease has not been studied in detail. The present study suggests that, at least for the role of alveolar macrophages, the early events in the pathogenesis of smoking-induced COPD mediated by AM in humans is unlikely inflammatory. Consistent with our observation of suppression of the M1 polarization pattern of human AM with smoking, other studies have observed decreased levels of transcripts for selected inflammatory cytokines in the BAL of smokers. For example, IL-6, CCL4, CCL5, CCL20 have been noted to be down-regulated in cells recovered by lavage of healthy smokers, although the cellular source of transcripts was not identified (44). Decreased expression of pro-inflammatory cytokines such as IL-6 and TNF-α as well as some surface molecules related to immune response in AM of healthy smokers has also been described (45–48). However, in general, these observations have been ignored in the context that exaggerated inflammatory and immune processes dominate in the lungs of patients with established COPD (7,9–12,41–43). The global gene expression analysis of AM carried out in the present study provides a transcriptome-based evidence that AM unlikely contribute to augmented production of inflammatory mediators in response to smoking, and is consistent with the basic concepts of macrophage polarization that not all forms of macrophage activation are pro-inflammatory (1–4).

Compatible with our observations, two previous studies in which the transcriptional pattern of AM of smokers was compared to nonsmokers have revealed that cigarette smoking is associated with induction of several genes related to tissue remodeling without activation of inflammatory program in AM, although the expression of macrophage polarization-related genes was not been addressed specifically (17,18). With regard to M1/M2-related genes, there is considerable overlap between these and our studies. Of 28 smoking-responsive M1- and M2-related genes identified in our study, 25 (89%) were detected in the study of Woodruff et al. (17) and 21 (75%) in the study of Heguy et al. (18). Among detected M1- and M2-related genes, 20 genes (80%) in the study of Woodruff et al (17) and 10 genes (47%) in the study of Heguy et al. (18) had the same direction of change as in our study (not shown). The overlapping genes, i.e. M1- and M2-related genes with the same direction of change, in these two studies and our study include M1-related genes TNFSF10, GBP1, PDE4B, and M2-related genes CCR5, CD9, and CD36. However, a direct gene-to-gene comparison of these studies is difficult due to different microarray analyzes used in these studies. Of interest, one of these studies demonstrated that AM of smokers displayed expression pattern distinct from those evoked in murine models of emphysema (17).

Second, from a host defense perspective, a broad suppression of inflammatory/immune genes in smokers is consistent with the epidemiologic data that smokers and COPD smokers are more susceptible to respiratory tract infection than nonsmokers (49). Accordant with this concept, AM of healthy smokers exhibit a decreased ability to kill intracellular bacteria *Listeria*

monocytogenes (50) and clearance of *Pseudomonas aeruginosa* is impaired in mice following chronic exposure to cigarette smoke (51). A decreased host defense potential of M1-deactivated AM resulting from smoking may, at least in part, explain why smokers who develop lung disease have an increased airway bacterial load and are frequently infected with respiratory viruses (52). A growing body of clinical evidence suggests that a variety of viruses such as rhinovirus, influenza virus, and respiratory syncytial virus are common causes of COPD exacerbations (52–54). It is, therefore, possible that in smokers with advanced COPD, persistent lung infection, which might develop due to deactivation of M1 polarization program in AM, initiates compensatory inflammatory responses, which are not directly induced by smoking. In support of this concept, cigarette smoking and viral components synergistically stimulate innate immune signaling in the mouse lung (55).

An important question that arises from these observations is how cigarette smoking might interfere with the M1 polarization program in AM? The direct immunosuppressive effect of various cigarette smoke constituents such as nicotine is well established (56). AM express the nicotinic acetylcholine receptor; when stimulated by nicotine, the AM support enhanced replication of intracellular bacteria and exhibit down-regulation of inflammatory cytokine production (57). Cigarette smoke extract inhibits the activation of nuclear factor kappa B (NF-kB), a master regulator of multiple inflammatory and immune processes in human macrophages (58). Acrolein, an aldehyde present in cigarette smoke, has been shown to decrease NF-kB activation in human AM (59). In contrast to the effects of chronic smoking on AM, acute exposure of macrophages to cigarette smoking extract, triggers the release of IL-8 and TNFα (60). The acute effect of cigarette smoking components on AM *in vitro* is likely very different from the effect of chronic smoking within the complex *in vivo* environment of human lung, involving multiple cell types. In this regard, the function of T cells, the major source of the M1-inducing cytokine IFNγ, is directly suppressed by cigarette smoke (56), and levels of IFNγ-producing T cells were found to be depressed in the lung of healthy smokers (61), consistent with decreased expression of IFNγ transcripts in cells recovered by lavage of smokers (44). Further, there are increased numbers of CD4+CD25+ regulatory T cells on the epithelial surface of healthy smokers (62,63). These regulatory T cells likely maintain an immunosuppressive microenvironment due to production of IL-10 and TGFβ (64), both inducing macrophage deactivation (3,21,22,65). In support of this scenario, the gene encoding integrin αE, important for induction of regulatory T cells in peripheral tissues (66), is up-regulated in AM of smokers (18). Another possibility is that chronic exposure to LPS, found in cigarette smoke at concentrations 120 times higher than levels found in smoke-free indoor air (67), induces inflammatory paralysis in AM, a phenomenon known as endotoxin tolerance (68). Consistent with this concept, stimulation of AM of smokers with LPS does not induce release of inflammatory cytokines comparable to that of AM of nonsmokers (69). Thus, a direct effect of smoke on AM as well as various changes in the cellular and cytokine microenvironment may account for cigarette smoking-induced deactivation of M1 polarization program in AM.

Induction of an Unusual M2 Polarization Program in AM by Cigarette Smoking

A broad deactivation of M1-polarization program in AM of healthy smokers was accompanied by induction of an unusual pattern of M2 polarization. This pattern was unique, in that the panel of up-regulated genes was not typical for any single M2 polarization program observed *in vitro*, but rather represented a mixture of different M2-related phenotypes. Smoking was associated with up-regulation of genes encoding scavenger receptors CD36, that can be induced in macrophages by IL-4 (29,70) or M-CSF (70), and MERTK, a receptor for apoptotic cells, that can be induced by glucocorticoids (31). AM of healthy smokers exhibited an increased expression of CCR5, a gene induced by IL-10 (71) and associated with the development of smoking-induced emphysema in mice (13,14). In addition, smoking increased expression of

MMP2, a gene associated with M-CSF-induced M2-like polarization program (72) and recently linked to the pathogenesis of COPD (73). In agreement with deactivated M1 gene expression pattern, the expression of CD9, a gene negatively regulated by IFNγ (33), was significantly increased in the AM of healthy smokers. Interestingly, induction of CD36 and CCR5 have been noted in two previous human studies in which the AM transcriptomes of healthy nonsmokers and smokers were compared (17,18).

Thus, cigarette smoking induces in AM a unique kind of M2-like polarization characterized by deactivation of M1 polarization program and induction of a diverse set of M2 polarization patterns, in a manner similar to M2-inducing immunosuppressive factors such as IL-10, TGFβ, and glucocorticoids, that do not only down-regulate the expression of M1-related genes, but also induce a special patterns of non-classical activation (3). However, the polarization state of AM in smokers was unique in that a broad M1 deactivation was accompanied by simultaneous activation of genes typical for various kinds of M2 polarization. Such pattern has not been described yet for any of known inducers of M2 macrophage phenotype. In the context that the M2 gene list used in our study was based on *in vitro* studies in which single cell-types (macrophages or monocytes) have been exposed to a defined, usually single, stimulus, our results suggest that the complex *in vivo* microenvironment generated in the lung of smokers dictates a novel macrophage polarization phenotype, distinct from those described *in vitro*.

It is not certain, however, whether induction of a such unusual M2-like phenotype in AM is due to abundance of known activators of M2-polarization program in the alveolar microenvironment of smokers. Although increased expression of IL-4 has been detected in human emphysematous lungs (42) and overexpression of both IL-4 and IL-13 in mice result in emphysema (13,74), decreased levels of IL-13 have been found in lavage fluid recovered from healthy smokers (44). While there are higher percentage of T cells expressing IL-4 and IL-13 in COPD smokers, these changes have not been observed in healthy smokers (75). Whether smoking modulates the levels of immunoregulatory cytokines IL-10 and TGFβ in the lung is also not certain, but the observation of increased numbers of regulatory T cells in the BAL of smokers (62,63) suggests such a possibility.

Heterogeneity of AM Responses to Smoking

Personalized assessment of AM transcriptomes revealed a diversity of biological phenotypes based on the response of AM polarization-related genes to smoking. Healthy smokers appeared to be a heterogeneous group in terms of expression of polarization-related genes; while ~70% cluster separately from nonsmokers, others expressed a pattern similar to healthy nonsmokers. These observations are consistent with epidemiological studies demonstrating that only a subset of smokers develop COPD (76) and lead to a hypothesis for future studies that subgroups of healthy smokers with a higher risk for the development of smoking-induced lung disease might be identified at the biologic level prior to the development of lung disease. Furthermore, the observed heterogeneity of AM responses to smoking suggests that the early pathogenetic mechanisms mediated by AM during the development of lung disease may be different in various subgroups of smokers, possibly resulting in distinct clinical phenotypes of the disease (77,78). Additional studies are necessary to determine whether healthy smokers whose AM are M1-deactivated will progress into a hypoinflammatory clinical phenotype of COPD. Indeed, it is well documented that anti-inflammatory therapy with corticosteroids is not effective in modifying the natural course of disease in subsets of patients with COPD (8–10).

Progressive Alteration of AM Polarization During the Development of COPD

The results of population-based comparative analysis utilized in our study indicate that transcriptional changes induced in AM by smoking have a progressive character, since M1-related genes were further down-regulated and M2-related genes were further up-regulated in

AM of COPD smokers when compared to smokers without disease. In AM of COPD smokers, there was advanced down-regulation of many host defense genes, including those encoding IFNγ-inducible chemokines CXCL9, CXCL10, CXCL11, and CCL5. The overall M1-related gene expression pattern in AM of COPD smokers was profoundly inhibited as compared to healthy nonsmokers and further down-regulated as compared to healthy smokers. The progressive suppression of the host defense and M1 polarization program was accompanied by progressive induction of several M2-related genes including MMP2, MMP7, and ADORA3. The tissue remodeling potential of MMPs (6,9,13) and MMP2, in particular, has already been implicated in the pathogenesis of COPD (73). Recent studies have shown that signaling through ADORA3, the glucocorticoid-inducible anti-inflammatory receptor, increases metalloprotease activity of macrophages (79) and activates TGF-β-dependent pro-fibrotic pathway in the lung (80).

Taken together, these results of the present study suggest that chronic cigarette smoking reprograms the steady-state AM polarization toward M1-deactivated, partially M2-activated macrophages with increased tissue remodeling potential but decreased expression of genes related to inflammation and immunity. This observation differs from those obtained in animal studies, in which induction of M2-like proteases in AM during the development of smoking-associated emphysema, a major component of COPD, has been found to occur in a concert with inflammatory response, as, for example, in transgenic mice overexpressing IFNγ (13, 81). Paradoxically, chemokines CCL5, CXCL9, CXCL10, and CXCL11 shown to be progressively down-regulated at the transcriptional level in healthy smokers and COPD smokers in our study, have been recognized as key mediators of emphysema development in different mouse models (13–15).

There may be several explanations for these mice-human differences. First, although IFNγ does induce emphysema in the murine lung, it seems to be not necessary for emphysema development in mice, because emphysema can also develop in mice that lack functional T cells (82), a major source of IFNγ, and mice overexpressing IL-13, a cytokine inducing M2 macrophage polarization (3), also develop emphysema (74). Second, it is known that physiology of respiratory and immune systems in rodents have a number of significant differences from human and animal models may not always reflect the features of human disease (42). Consistent with this, there are substantial differences among the AM gene signatures of human smokers and two transgenic models of emphysema (17).

Finally, there are several other cell types than AM in the lung capable of producing inflammatory mediators (9,10,39). In this regard, several lines of evidence suggest that lung T cells activated during the development of smoking-induced lung disease may serve as source of IFNγ-inducible chemokines, such as CXCL9, CXCL10 and CXCL11(15,16), known to be associated with M1 macrophage polarization (4,29) and found to be suppressed in AM of healthy smokers and COPD smokers in our study. Elevated levels of CCL5, CXCL9, CXCL10, CXCL11 detected in the induced sputum of COPD smokers in one recent study correlated positively with neutrophil but negatively with macrophage numbers (83) and increased expression of CXCL10 was found in the bronchiolar epithelium and pulmonary arteries in smokers with COPD (84). Studies in transgenic mice and lung cells obtained from COPD patients have revealed that these chemokines are responsible for inducing protease activity in lung macrophages increasing thereby their tissue damaging potential (13,15,16). However, it remains unclear why such IFNγ-dependent mechanism does not induce M1 polarization program in AM. It is possible that inflammatory mechanisms unraveled in that studies are more characteristic for advanced stages of COPD pathogenesis. The results of the present study suggest that early mechanisms of smoking-induced lung disease in humans are likely highlighted by a complex suppression of various aspects of immune response in the lung,

including deactivation of AM inflammatory and host defense function, and development of tissue remodeling processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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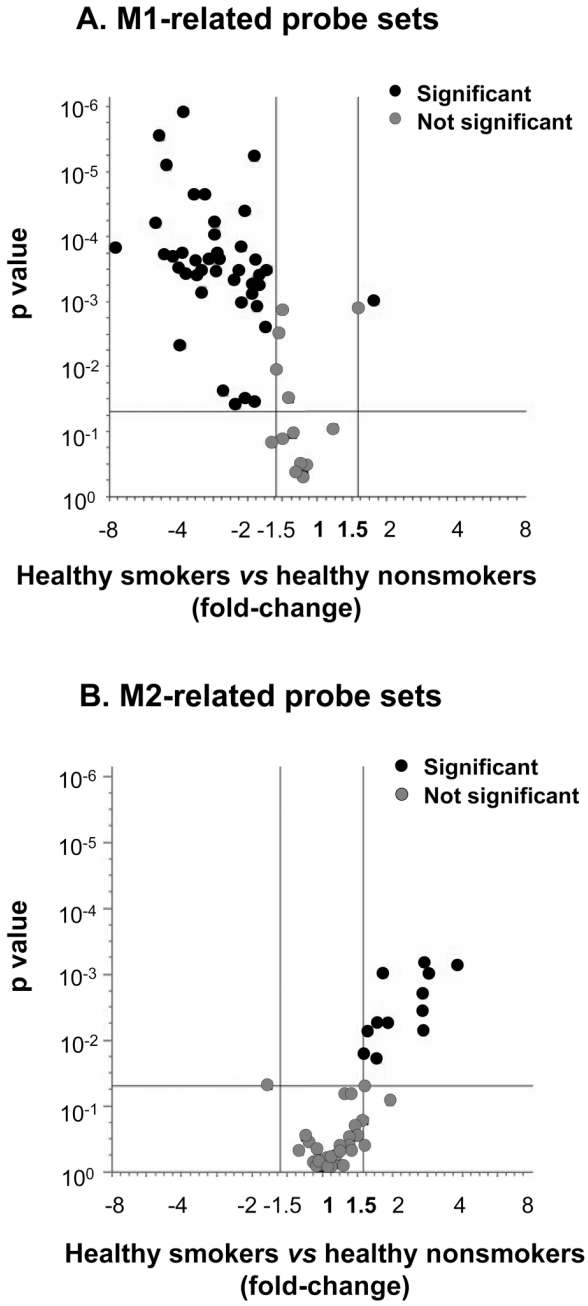

Figure 1.

Smoking-mediated reciprocal induction of M1 and M2 polarization programs of human alveolar macrophages. **A**. Volcano plot of M1-related probe sets significantly differently expressed between healthy nonsmokers (n=24) and healthy smokers (n=34). **B**. Volcano plot for the M2-related gene probe sets comparing the same groups. For both panels, the x-axis corresponds to the fold-change and the y-axis corresponds to p value. Red dots represent significant differentially expressed probe sets, grey dots represent probe sets with no significant difference between healthy smokers and healthy nonsmokers. The changes in gene expression were considered statistically significant based on the criteria of fold-change ≥1.5, $p<0.05$ with Benjamini-Hochberg correction.

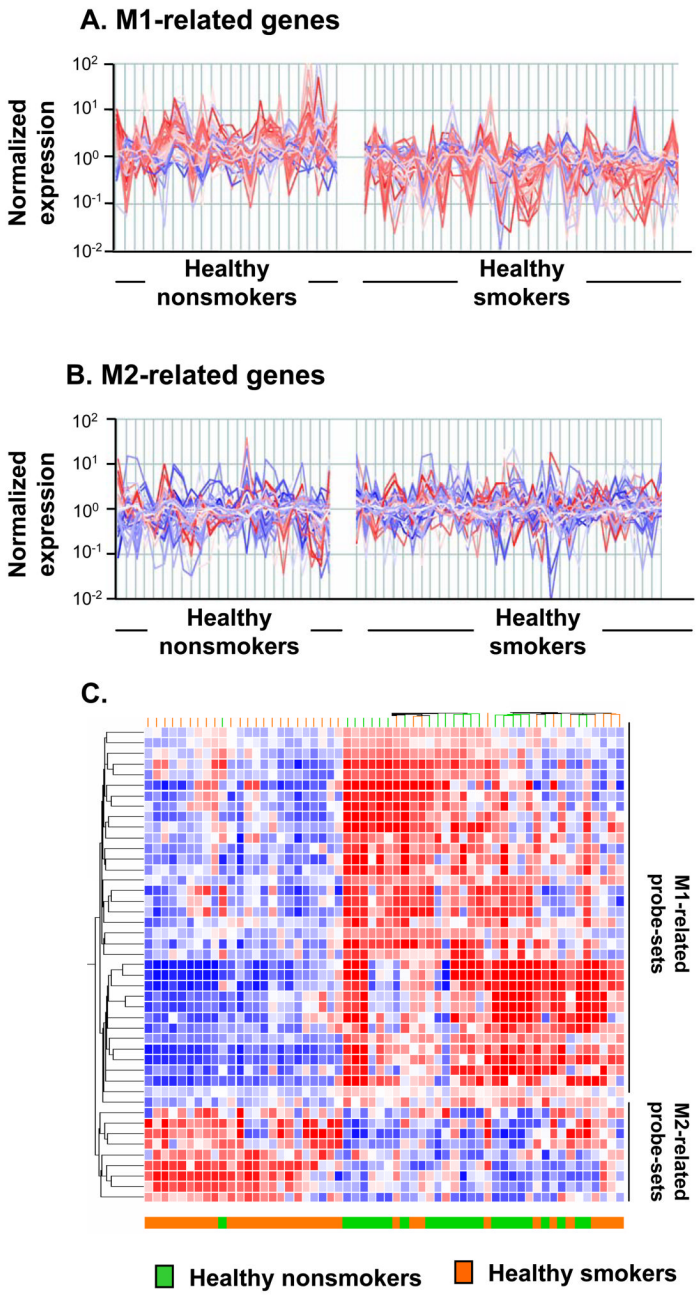

Figure 2.

Biologic phenotypes of healthy smokers compared to healthy nonsmokers based on alveolar macrophage M1- and M2-related gene expression. **A**. Expression of all M1-related probe sets in AM of healthy nonsmokers (n=24; left panels) compared to that of healthy smokers (n=34; right panels). See Table II for a list of all M1-related probes. **B**. Expression of all M2-related probe sets in AM comparing the same groups (healthy nonsmokers, left; healthy smokers, right). See Table III for a list of all M2-related probes. For both **A** and **B**, the y-axis indicates normalized relative expression levels for the probe sets, the x-axis shows the individuals belonging to each group randomly ordered but of similar order in **A** and **B**. Red = gene probe sets down-regulated in healthy smokers as compared to healthy nonsmokers; blue = gene probe

sets up-regulated in healthy smokers as compared to healthy smokers. Intensity of color indicates the degree of down- or up-regulation. Note that overall, the M1-related genes tend to be down-regulated in the healthy smokers compared to the healthy nonsmokers. The opposite is observed among the M2-related genes, but not to the same extent as the down-regulation of the M1 genes. **C**. Non-supervised hierarchical cluster analysis of AM M1- and M2-related gene expression of healthy nonsmokers and healthy smokers. The analysis is based on healthy smokers, the differential expression of M1- and M2- related genes of the same groups of healthy nonsmokers (n=24) and healthy smokers (n=34) using Spearman correlation as a similarity measure and an average linkage as a clustering algorithm. Statistically significant differentially expressed M1- and M2-gene probe sets were used as input data set. Genes expressed above average are represented in red, below average in blue, and average in white. The genes are represented vertically, and individual subjects horizontally at the bottom. Healthy nonsmokers are indicated by green, healthy smokers by orange. Although, there is variability within each group, compared to the healthy nonsmokers, the general tendency for the healthy smokers is for the M1 genes to be down-regulated and the M2 genes up-regulated.

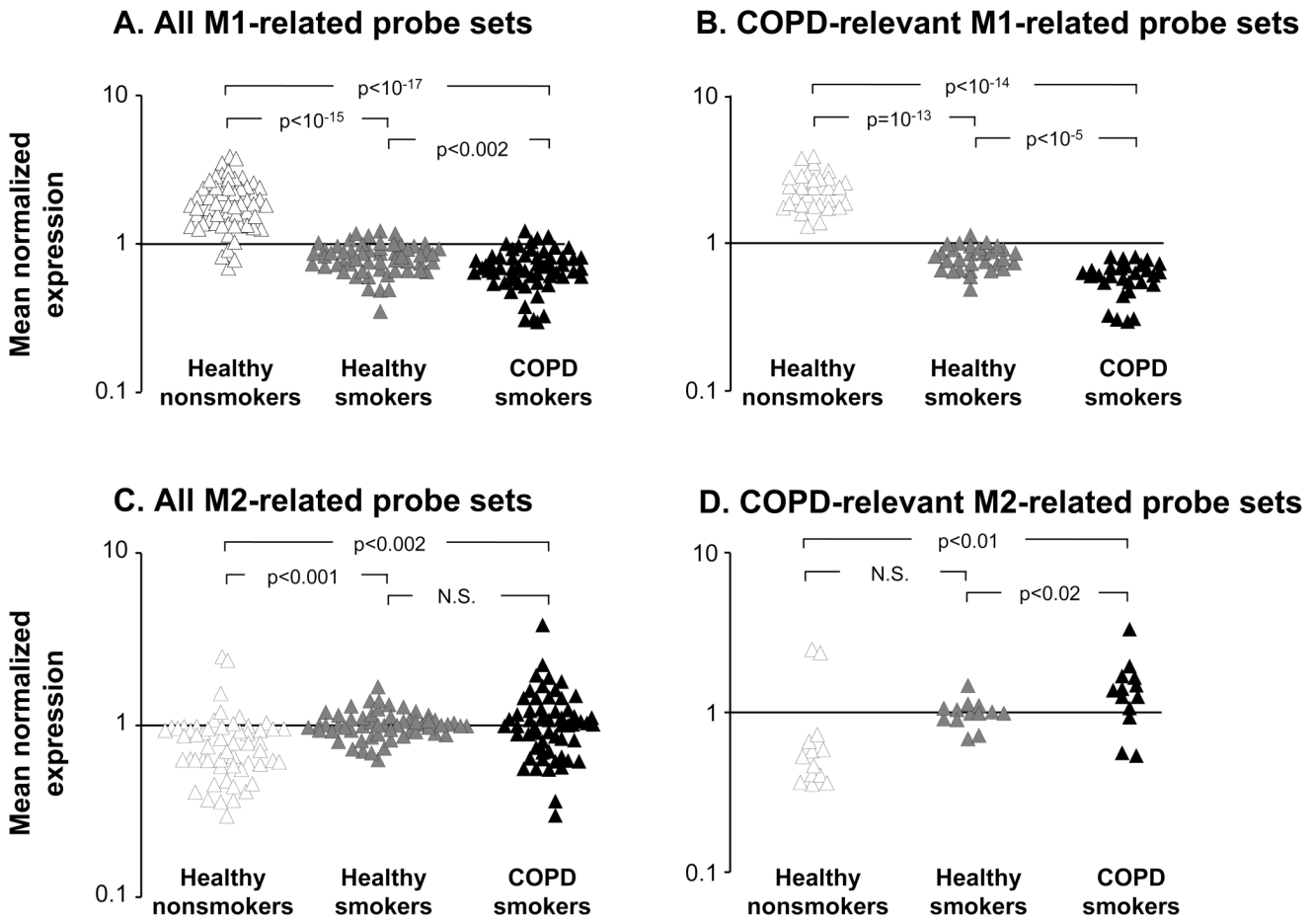

Figure 3.

Progressive reciprocal alteration of M1- and M2-related gene expression in alveolar macrophages with the development of COPD. **A**. Normalized expression levels of all M1-related probe sets; **B**. M1-related probe sets significantly differentially expressed in AM of COPD smokers *vs* healthy nonsmokers; **C**. All M2-related probe sets; and **D**. M2-related probe sets significantly differentially expressed in AM of COPD smokers *vs* healthy nonsmokers. The data is based on healthy nonsmokers (n=24), healthy smokers (n=34) and COPD smokers (n=12). The y-axis indicates mean normalized, expression levels for the probe sets in each group; the x-axis indicates the groups. p values represent differences among groups as indicated.

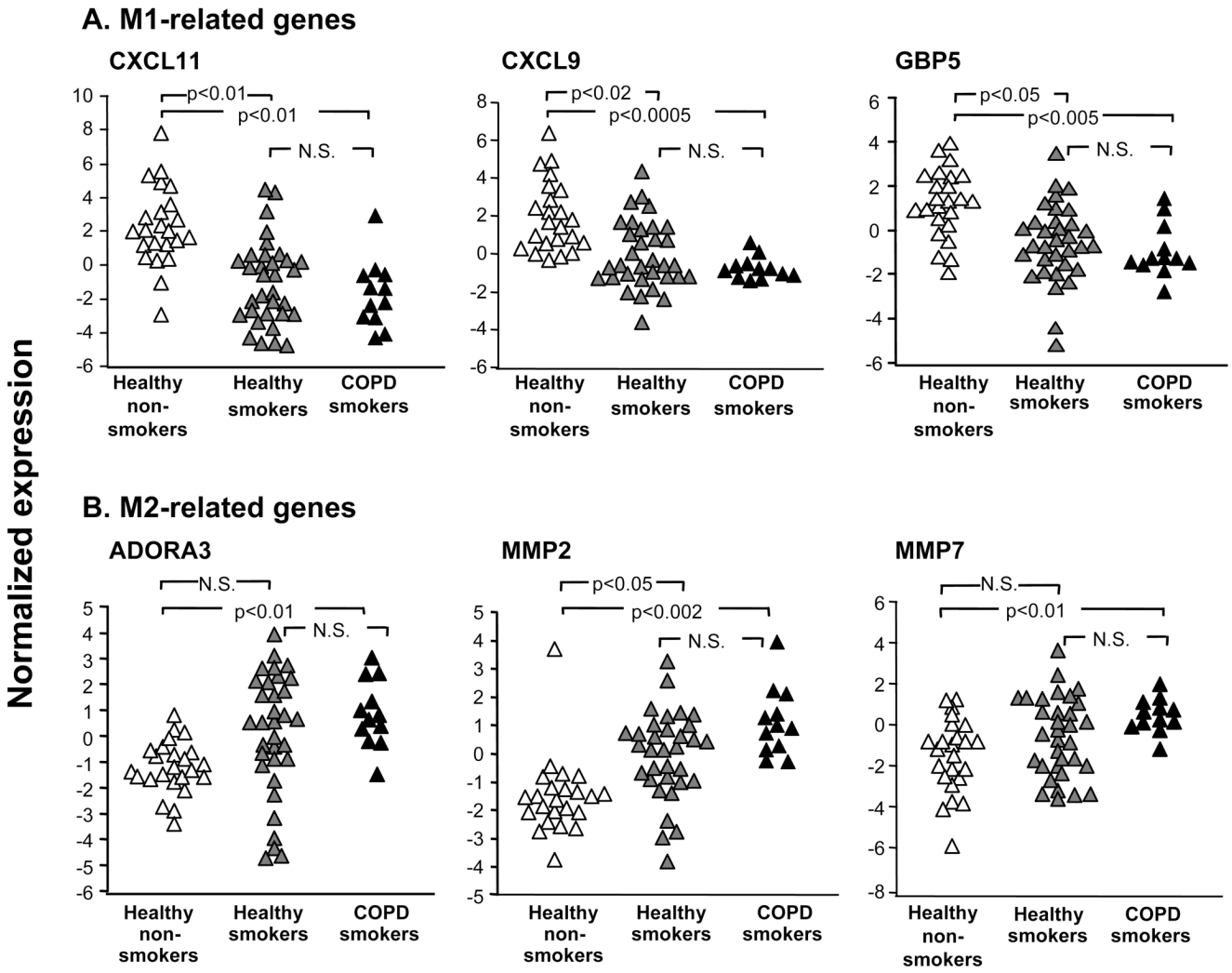

Figure 4.

Examples of AM expression of M1 and M2 polarization-related genes demonstrating progressive differences with the development of COPD. **A**. M1-related genes; and **B**. M2-related genes. Log2-transformed normalized expression levels for selected M1-related genes [C-X-C chemokine ligand 11 (CXCL11); C-X-C chemokine ligand 9 (CXCL9); guanylate binding protein (GBP 5)]; and M2-related genes [adenosine A3 receptor (ADORA3); matrix metalloprotease 2 (MMP2); and matrix metalloproteinase 7 (MMP7), are plotted for all healthy nonsmokers (n=24; green triangles), healthy smokers (n=32; orange triangles), and COPD smokers (n=12; blue triangles). p values are indicated. N.S., non-significant.

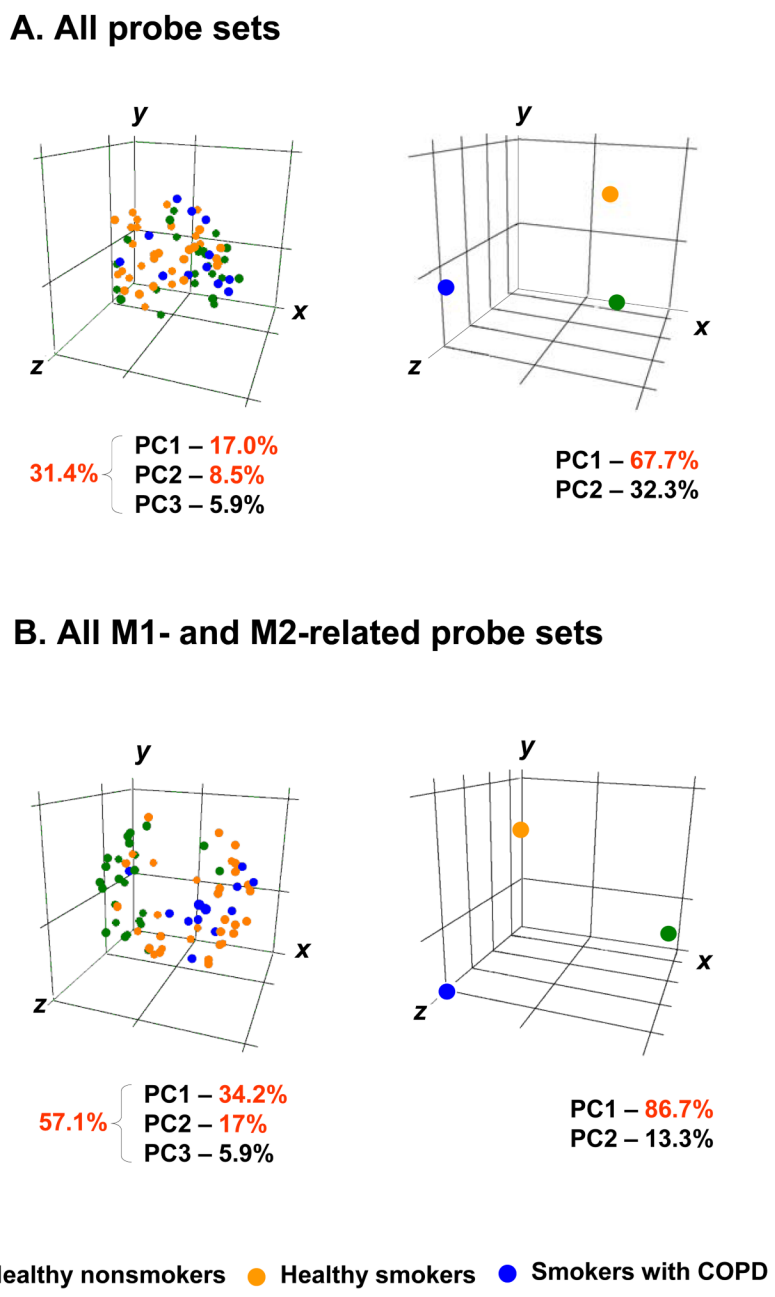

Figure 5.

Principle component analysis (PCA) comparison of alveolar macrophage gene expression patterns of healthy smokers, healthy smokers and COPD smokers. **A**. PCA of all 26,959 probe sets expressed in AM of at least 20% of subjects. **B**. PCA of all 114 M1- and M2-related probe sets expressed in AM of at least 20% of subjects (see Tables I, II). In both analyses, all samples within each group were centered (left panels) or averaged and then the means were centered (right panels) in the three-dimensional space based on the expression pattern. In left panels, each circle represents an individual sample; in right panels, each circle represents an averaged sample for each group. Healthy nonsmokers, n=24, green; healthy smokers, n=34, orange; and COPD smokers, n=12, blue). The percentage contributions of the first three (left panels) or

two (right panels) principal components (PC) to the observed variability between the groups are indicated.

Table I

Demographic Characteristics of the Study Population and Biologic Samples[1, 2]

Parameter	Healthy nonsmokers	Healthy smokers	COPD smokers
Number	24	34	12
Sex (male / female)	18/6	26/8	10/2
Age (yr)	40.3 ± 8.2	41.3 ± 7.9	54.7 ± 9.3*, **
Ancestry (B/W/H[3])	15/6/3	20/10/4	1/8/3
Smoking history (pack-yr)	0	26.8 ± 17.7	49.8 ± 28.3**
Urine nicotine (ng/ml)	negative	864 ± 1053	1217 ± 1301
Urine cotinine	negative	1211 ± 1034	1243 ± 639
Venous carboxyhemoglobin (%)[4]	0.5 ± 0.8	3.0 ± 2.1	3.2 ± 2.1
Pulmonary function[5]			
FVC	106.1 ± 11.2	107.0 ± 10.8	105.2 ± 24.6
FEV1	102.9 ± 11.8	108.0 ± 13.2	85.2 ± 22.9*, **
FEV1/FVC	81.5 ± 4.7	81.9 ± 4.9	64.2 ± 4.4*, **
TLC	96.8 ± 8.1	99.0 ± 12.6	112.8 ± 21.6*, ****
DLCO	94.3 ± 8.5	96.0 ± 11.2	75.4 ± 17.0*, **
GOLD Stage (I/II/III)[6]	--	--	8/3/1

[1] All data are mean ± standard deviation.

[2] For the criteria for the study groups, see Methods.

[3] B, black; W, white; H, hispanic.

[4] Venous carboxyhemoglobin, a secondary marker of current smoking; normal level for nonsmokers <1.5%.

[5] FVC - forced vital capacity; FEV1- forced expiratory volume in 1 sec; DLCO - diffusing lung capacity for carbon monoxide; TLC - total lung capacity; all values are presented as % predicted except for FEV1/FVC presented as % observed.

[6] COPD staging defined by the GOLD (the Global Initiative for Chronic Obstructive Lung Disease) criteria (7).

* $p<0.05$ as compared to healthy nonsmokers

** $p<0.05$ as compared to healthy smokers.

Table II

M1-related Gene Expression in Alveolar Macrophages of Healthy Nonsmokers and Healthy Smokers and COPD-smokers[1, 2]

Gene name	Ref	Symbol	Probeset ID	P call (%)			Fold-change			p value			M profile[3]		
				NS	S	COPD-S	S *vs* NS	COPD-S *vs* NS	COPD-S *vs* S	S *vs* NS	COPD-S *vs* NS	COPD-S *vs* S	S *vs* NS	COPD-S *vs* NS	COPD-S *vs* S
Membrane receptors															
Fc fragment of IgG, low affinity IIIa, receptor (CD16a)	(2–4,23)	FCGR3A	204006_s_at	100	100	100	−1.1	−1.8	−1.5	N.S.	N.S.	N.S.	-	-	-
Fc fragment of IgG, low affinity IIa, receptor (CD32)	(2–4,23)	FCGR2A	203561_at	100	100	100	−1.5	−1.4	1	N.S.	N.S.	N.S.	-	-	-
			1565674_at	29	27	42	−1.2	1.3	1.5	N.S.	N.S.	N.S.			
Fc fragment of IgG, high affinity Ia, receptor (CD64)	(2–4,23)	FCGR1A	216950_s_at	100	100	100	−1.2	−1.2	1	N.S.	N.S.	N.S.	-	-	-
Interleukin 15 receptor, alpha	(29)	IL15RA	207375_s_at	100	100	100	−1.6	−1.7	−1.1	<0.02	<0.02	N.S.	↓	↓	-
Complement component 3a receptor 1	(23,26)	C3AR1	209906_at	100	100	100	−1.8	−1.7	1.1	<0.03	N.S.	N.S.	↓	-	-
CD69 antigen (p60, early T-cell activation antigen)	(23)	CD69	209795_at	83	27	8	−4.8	-10	−2.1	<0.0003	<0.01	N.S.	↓	↓	-
CD80 antigen (CD28 antigen ligand 1, B7-1 antigen)	(2–4,23)	CD80	1554519_at	100	100	100	−1.9	−1.6	1.2	<0.03	N.S.	N.S.	↓	-	-
			1555689_at	96	47	92	−3.3	−1.3	2.5	<0.002	N.S.	<0.03			
CD86 antigen (CD28 antigen ligand 2, B7-2 antigen)	(2–4,23)	CD86	205686_s_at	100	100	100	1.2	1	−1.2	N.S.	N.S.	N.S.	-	-	-
Toll-like receptor 2	(4)	TLR2	204924_at	100	100	100	−1.6	−1.8	−1.1	N.S.	N.S.	N.S.	-	-	-
Toll-like receptor 4	(4)	TLR4	1552798_a_at	100	100	100	1.8	1	−1.5	<0.04	N.S.	<0.03	↑	-	↓
			221060_s_at	100	100	100	1.5	−1.1	−1.6	<0.05	N.S.	N.S.			
Intercellular adhesion molecule 1 (CD54)	(26)	ICAM1	215485_s_at	96	88	92	−1.4	−3.3	−2.4	N.S.	<0.005	<0.02	-	↓	↓
Cytokines and chemokines															
Interleukin 1, beta	(2)	IL1B	205067_at	100	100	100	−2.3	−3.3	−1.4	<0.03	<0.01	N.S.	↓	↓	-
			39402_at	100	100	100	−2.3	−3.1	−1.3	<0.02	<0.01	N.S.			
Interleukin 6 (interferon, beta 2)	(2,29)	IL6	205207_at	75	56	67	−2.2	−1.7	1.3	N.S.	N.S.	N.S.	-	-	-
Interleukin 12B (natural killer cell stimulatory factor 2, p40)	(2,3,29)	IL12B	207901_at	21	3	17	−2.1	−1.2	1.7	N.S.*	N.S.*	N.S.*	-	-	-
Interleukin 18 (interferon-gamma-inducing factor)	(85)	IL18	206295_at	100	100	100	−1.9	−1.7	1.1	<0.0007	N.S.	N.S.	↓	-	-

Gene name	Ref	Symbol	Probeset ID	P call (%)			Fold-change			p value			M profile[3]		
				NS	S	COPD-S	S *vs* NS	COPD-S *vs* NS	COPD-S *vs* S	S *vs* NS	COPD-S *vs* NS	COPD-S *vs* S	S *vs* NS	COPD-S *vs* NS	COPD-S *vs* S
Interleukin 23, alpha subunit p19	(4)	IL23	220054_at	42	27	33	−1.6	−2.1	−1.4	N.S.	N.S.	N.S.	-	-	-
Interleukin 32	(86)	IL32	203828_s_at	13	6	0	−5.2	−17.1	−3.3	<0.003*	<0.0002*	<0.05*	↓	↓	↓
Tumor necrosis factor (TNF superfamily, member 2)	(2,29)	TNF	207113_s_at	100	100	100	−1.2	−2.1	−1.8	N.S.	N.S.	N.S.	-	-	-
Tumor necrosis factor (ligand) superfamily, member 10	(29)	TNFSF10	202687_s_at	96	68	42	−2.9	−3.7	−1.3	<0.02	<0.02	N.S.	↓	↓	-
			202688_at	96	91	92	−3.2	−3.4	−1.1	<0.02	<0.02	N.S.			
			214329_x_at	83	50	92	−3.4	−2.2	1.6	<0.02	N.S.	N.S.			
Tumor necrosis factor, alpha-induced protein 6	(29)	TNFAIP6	206025_s_at	100	100	92	−4.6	−7.9	−1.7	<0.01	<0.02	N.S.	↓	↓	-
			206026_s_at	100	100	100	−4.9	−5.4	−1.1	<0.004	<0.03	N.S.			
Chemokine (C-X-C motif) ligand 1	(23)	CXCL1	204470_at	100	100	100	−3.1	−2.6	1.2	<0.03	N.S.	N.S.	↓	-	-
Chemokine (C-X-C motif) ligand 9	(4,29)	CXCL9	203915_at	100	62	75	−4	−6.5	−1.6	<0.02	<0.0005	N.S.	↓	↓	-
Chemokine (C-X-C motif) ligand 10	(4,29)	CXCL10	204533_at	83	47	17	−3.8	−5.3	−1.4	N.S.	<0.05	N.S.	-	↓	-
Chemokine (C-X-C motif) ligand 11	(4,29)	CXCL11	211122_s_at	63	18	8	−7.7	−11.5	−1.5	<0.01	<0.01	N.S.	↓	↓	-
			210163_at	63	21	8	−4.1	−5	−1.2	<0.01	<0.05	N.S.			
Chemokine (C-C motif) ligand 4	(4,26)	CCL4	204103_at	100	100	100	−3.3	−3.7	−1.1	<0.02	N.S.	N.S.	↓	-	-
Chemokine (C-C motif) ligand 5	(4,29)	CCL5	1405_i_at	92	68	50	−3.8	−4.7	−1.2	<0.01	<0.01	N.S.	↓	↓	-
		CCL5	1555759_a_at	75	35	25	−2.8	−3.5	−1.3	<0.01	<0.008	N.S.			
			204655_at	92	65	75	−2.8	−2.5	1.1	<0.005	<0.05	N.S.			
Chemokine (C-C motif) ligand 20	(4,29)	CCL20	205476_at	100	85	83	−2.5	−5.8	−2.3	N.S.	N.S.	N.S.	-	-	-
Signaling related proteins															
Guanylate binding protein 1, interferon-inducible,	(23,26)	GBP1	202270_at	100	100	100	−2.2	−2	1.1	<0.02	N.S.	N.S.	↓	-	-
67 kDa			231577_s_at	100	100	100	−1.9	−2.5	−1.3	N.S.	N.S.	N.S.			
			231578_at	67	50	75	−2	−1.2	1.7	N.S.	N.S.	N.S.			
Guanylate binding protein 2, interferon-inducible	(23,26)	GBP2	202748_at	100	100	100	−1.9	−2.7	−1.5	<0.02	<0.0005	<0.04	↓	↓	↓
			242907_at	100	100	100	−2.2	−2.1	1	<0.002	<0.03	N.S.			
Guanylate binding protein 3	(26)	GBP3	223434_at	100	100	100	−1.5	−1.4	1.1	N.S.	N.S.	N.S.	-	-	-
Guanylate binding protein 4	(26)	GBP4	235175_at	96	56	25	−2.1	−3	−1.4	<0.01	<0.002	N.S.	↓	↓	-
			235574_at	79	44	0	−2	−6	−3.1	N.S.	<0.0006	<0.02			

Gene name	Ref	Symbol	Probeset ID	P call (%) NS	P call (%) S	P call (%) COPD-S	Fold-change S *vs* NS	Fold-change COPD-S *vs* NS	Fold-change COPD-S *vs* S	p value S *vs* NS	p value COPD-S *vs* NS	p value COPD-S *vs* S	M profile[3] S *vs* NS	M profile[3] COPD-S *vs* NS	M profile[3] COPD-S *vs* S
Guanylate binding protein 5	(26)	GBP5	229625_at	96	68	42	−2.7	−3.4	−1.3	<0.05	<0.003	N.S.	↓	↓	-
			238581_at	88	50	58	−3.1	−3	1	<0.01	<0.01	N.S.			
Immunoresponsive 1 homolog	(23,26)	IRG1	240287_at	79	24	17	−4.1	−4.2	1	<0.001	<0.004	N.S.	↓	↓	-
Interferon regulatory factor 1	(23,26,29)	IRF1	202531_at	100	100	100	−1.3	−1.9	−1.5	N.S.	N.S.	N.S.	-	-	-
Interferon regulatory factor 7	(23,26,29)	IRF7	208436_s_at	100	100	100	−1.3	−1.7	−1.3	N.S.	<0.02	N.S.	-	↓	-
Suppressor of cytokine signaling 3	(87)	SOCS3	227697_at	100	97	100	−2.7	−3.1	−1.2	<0.02	<0.03	N.S.	↓	↓	-
			206359_at	50	35	0	−1.9	−6	−3.1	N.S.	<0.02	<0.05			
Enzymes and other proteins															
Nitric oxide synthase 2A (inducible)	(3,23,26)	NOS2A	210037_s_at	0	0	0	N.D.**	N.D.**	N.D.**	N.S.*	N.S.*	N.S.*	-	-	-
Phosphodiesterase 4B, cAMP-specific	(23)	PDE4B	203708_at	100	97	92	−3.7	−3.7	1	<0.0002	<0.05	N.S.	↓	↓	-
			211302_s_at	100	100	100	−3.2	−4.5	−1.4	<0.002	<0.02	N.S.			
			215671_at	54	15	33	−3.8	−2.6	1.5	<0.02	N.S.	N.S.			
			222326_at	83	44	50	−4.4	−3.1	1.4	<0.01	N.S.	N.S.			
Apolipoprotein L, 3	(29)	APOL3	221087_s_at	100	100	92	−1.8	−2	−1.2	<0.03	<0.003	N.S.	↓	↓	-
B-factor, properdin (complement factor B)	(26)	CFB	202357_s_at	96	85	50	−2.7	−3.9	−1.4	<0.005	<0.0005	N.S.	↓	↓	-

[1] Genes listed as M1-related if there is evidence in literature that they are induced in mononuclear phagocytes by IFNγ and / or LPS. Genes induced by both M1 and M2 macrophage stimuli were not included; see text for details.

[2] Abbreviations: NS, healthy nonsmokers; S, healthy smokers; N.S., non-significant.

[3] "M profile" of healthy smokers *vs* healthy nonsmokers, COPD smokers *vs* nonsmokers, and COPD smokers *vs* healthy smokers are indicated (↑ = up-regulated; - = no change; ↓ = down-regulated, p<0.05).

* probe sets with very low expression level (average P call < 20%)

Table III

M2-related Gene Expression in AM of Healthy Nonsmokers and Healthy Smokers[1, 2]

				P call (%)			Fold-change			p value			M profile[3]		
Gene name	**Ref**	**Symbol**	**Probe set ID**	**NS**	**S**	**COPD-S**	**S *vs* NS**	**COPD-S *vs* NS**	**COPD-S *vs* NS**	***Svs* NS**	**COPD-S *vs* NS**	**COPD-S *vs* NS**	**S *vs* NS**	**COPD-S *vs* NS**	**COPD-S *vs* NS**
Membrane receptors															
Macrophage scavenger receptor 1	(3,29)	MSR1	208422_at	100	100	100	1.1	−1.7	−1.8	N.S.	N.S.	N.S.	-	-	-
			208423_s_at	100	100	100	1	−1.1	−1.2	N.S.	N.S.	N.S.			
Mannose receptor, C type 1	(2,3,29)	MRC1	204438_at	100	100	100	1	1.1	1	N.S.	N.S.	N.S.	-	-	-
Mannose receptor, C type 2	(2,3)	MRC2	37408_at	13	47	25	1.5	1.9	1.3	N.S.	N.S.	N.S.	-	-	-
Chemokine (C-X-C motif) receptor 4	(4,29)	CXCR4	217028_at	100	100	100	1.1	1.4	1.3	N.S.	N.S.	N.S.	-	-	-
			211919_s_at	96	100	100	1.2	1	−1.2	N.S.	N.S.	N.S.			
Chemokine (C-C motif) receptor 5	(3,71)	CCR5	206991_s_at	100	100	100	1.9	1.6	−1.2	<0.02	N.S.	N.S.	↑	-	-
C-type lectin domain family 7A	(2,88)	CLEC7A	221698_s_at	100	100	100	−1.2	1	1.2	N.S.	N.S.	N.S.	-	-	-
			241098_at	42	50	92	1.3	2.3	1.7	N.S.	N.S.	N.S.			
			1554406_a_at	100	100	100	1	−1.2	−1.2	N.S.	N.S.	N.S.			
			1555214_a_at	88	100	92	1.1	−1.6	−1.8	N.S.	N.S.	N.S.			
			1555756_a_at	100	100	100	1.1	1	−1.2	N.S.	N.S.	N.S.			
Purinergic receptor P2Y, G-protein coupled, 5	(29)	P2RY5	218589_at	100	100	100	1.5	2.1	1.3	N.S.	<0.02	N.S.	-	↑	-
Stabilin 1	(89)	STAB1	38487_at	13	53	67	1.8	2.1	1.2	N.S.	N.S.	N.S.	-	-	-
CD9 antigen (p24)	(33)	CD9	233317_at	4	38	25	3.9	1.9	−2	<0.01	N.S.	N.S.	↑	-	-
CD36 (collagen type I receptor)	(29,70)	CD36	241929_at	88	100	100	2.9	3	1	<0.02	<0.03	N.S.	↑	↑	-
			228766_at	100	100	100	1.9	2.2	1.1	<0.05	N.S.	N.S.			
			236923_x_at	100	100	100	1.7	2.5	1.4	N.S.	<0.01	N.S.			
			206488_s_at	100	100	100	1.5	1.6	1.1	N.S.	N.S.	N.S.			
			209554_at	8	27	17	−1.1	1.2	1.4	N.S.	N.S.	N.S.			
			209555_s_at	100	100	100	1.6	1.4	-1.1	N.S.	N.S.	N.S.			

Gene name	Ref	Symbol	Probe set ID	P call (%)			Fold-change			p value			M profile[3]		
				NS	S	COPD-S	S *vs* NS	COPD-S *vs* NS	COPD-S *vs* NS	*S*vs NS	COPD-S *vs* NS	COPD-S *vs* NS	S *vs* NS	COPD-S *vs* NS	COPD-S *vs* NS
			242197_x_at	42	85	83	2.9	2.3	-1.3	<0.01	N.S.	N.S.			
CD163 antigen	(2,3)	CD163	203645_s_at	100	100	100	1	1.2	1.1	N.S.	N.S.	N.S.	-	-	–
			215049_x_at	100	100	100	1.2	1.2	1.2	N.S.	N.S.	N.S.			
			216233_at	83	91	92	1.2	1.5	1.2	N.S.	N.S.	N.S.			
c-mer proto-oncogene tyrosine kinase	(31)	MERTK	206028_s_at	54	91	100	2.7	4.7	1.7	<0.04	<0.004	N.S.	↑	↑	-
			211913_s_at	54	91	100	2.7	3	1.1	<0.006	<0.03	N.S.			
Adenosine A3 receptor	(31)	ADORA3	223660_at	13	56	83	2.7	4.6	1.7	N.S.	<0.01	N.S.	-	↑	-
			206171_at	21	53	50	2.1	3.1	1.5	N.S.	N.S.	N.S.			
Fc fragment of IgE, low affinity II, receptor for (CD23)	(2,3)	FCER2	206759_at	0	0	0	N.D.**	N.D.**	N.D.**	N.S.*	N.S.*	N.S.*	-	-	-
Interleukin 4 receptor	(3)	IL4R	203233_at	100	100	92	−1.1	−1.2	−1.1	N.S.	N.S.	N.S.	-	-	-
			242743_at	58	82	67	1.3	1.4	1.1	N.S.	N.S.	N.S.			
Cytokines and chemokines															
Interleukin 10	(2–4)	IL10	207433_at	54	50	58	1	1.7	1.7	N.S.	N.S.	N.S.	-	-	-
Interleukin 1 receptor antagonist	(2–4)	IL1RN	212657_s_at	100	100	100	1	−1.4	−1.3	N.S.	N.S.	N.S.	-	-	-
			212659_s_at	100	100	100	1.3	−2.6	−3.5	N.S.	N.S.	N.S.			
			216243_s_at	100	100	100	1.4	−3.3	−4.4	N.S.	N.S.	<0.006			
Transforming growth factor, beta 1	(3)	TGFB1	203085_s_at	100	100	100	1.4	−1.2	−1.7	N.S.	N.S.	N.S.			
Chemokine (C-C motif) ligand 17	(4)	CCL17	207900_at	25	0	0	−1.2	N.D.**	N.D.**	N.S.*	N.S.	N.S.	-	-	-
Chemokine (C-C motif) ligand 18	(2–4,29)	CCL18	32128_at	100	100	100	−1.2	1.1	1.3	N.S.	N.S.	N.S.	-	-	-
			209924_at	100	100	100	−1.1	1.1	1.2	N.S.	N.S.	N.S.			
Chemokine (C-C motif) ligand 22	(4)	CCL22	207861_at	54	50	42	1.4	−1.1	−1.6	N.S.	N.S.	N.S.	-	-	-
Chemokine (C-C motif) ligand 23	(29,40)	CCL23	210548_at	100	97	92	−2.5	−3.9	−1.6	N.S.	<0.02	N.S.	-	↓	-
			210549_s_at	100	97	92	−2.4	−3.9	−1.6	N.S.	<0.03	N.S.			
Chemokine (C-C motif) ligand 24	(4)	CCL24	221463_at	54	68	8	1.2	−2.1	−2.5	N.S.	N.S.	N.S.	-	-	-
Signaling-related proteins															

				P call (%)			Fold-change			p value			M profile[3]		
Gene name	**Ref**	**Symbol**	**Probe set ID**	**NS**	**S**	**COPD-S**	**S *vs* NS**	**COPD-S *vs* NS**	**COPD-S *vs* NS**	***S*vs NS**	**COPD-S *vs* NS**	**COPD-S *vs* NS**	**S *vs* NS**	**COPD-S *vs* NS**	**COPD-S *vs* NS**
Regulator of G-protein signaling 1	(32)	RGS1	216834_at	100	100	100	1	2.6	2.5	N.S.	<0.005	<0.008	-	↑	-
Growth arrest-specific 7	(29)	GAS7	202191_s_at	92	100	100	2.1	3.3	1.6	N.S.	<0.02	N.S.	-	↑	-
			211067_s_at	75	82	100	1.8	1.9	1.1	N.S.	N.S.	N.S.			
Enzymes and other proteins															
Arginase, liver	(2,3,38)	ARG1	206177_s_at	17	24	0	1	N.D.**	N.D.**	N.S.*	N.S.*	N.S.*	-	-	-
Matrix metallopeptidase 2 (gelatinase A, 72 kDa gelatinase)	(72)	MMP2	201069_at	63	88	100	2.7	6.2	2.2	<0.03	<0.002	N.S.	↑	↑	-
			1566678_at	29	21	42	−1.4	1	1.4	N.S.	N.S.	N.S.			
Matrix metallopeptidase 7 (matrilysin, uterine)	(27)	MMP7	204259_at	25	50	58	2	3.9	2	N.S.	<0.009	N.S.	-	↑	-
Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase)	(3)	MMP9	203936_s_at	100	100	100	1.4	1.7	1.2	N.S.	N.S.	N.S.	-	-	-
Heparan sulfate (glucosamine) 3-0-sulfotransferase 1	(29)	HS3ST1	205466_s_at	42	71	67	2.2	3.2	1.4	N.S.	N.S.	N.S.	-	-	-
Heparan sulfate (glucosamine) 3-0-sulfotransferase 2	(29)	HS3ST2	219697_at	83	79	100	1.5	5.2	3.6	N.S.	<0.006	<0.02	-	↑	↑
Collagen, type VI, alpha 2	(90)	COL6A2	209156_s_at	38	50	42	1.6	1.1	−1.4	N.S.	N.S.	N.S.	-	-	-
Fibronectin	(29,91)	FN1	214701_s_at	100	100	92	2.4	1.3	1.8	N.S.	N.S.	N.S.	-	-	-

[1] Genes listed as M2-related if there is evidence in literature that they are induced in mononuclear phagocytes by IL-4 and IL-13 (alternative activation), IL-10 (deactivation), TGF-β, glucocorticoids, M-CSF (see references given for every gene); genes induced by both M1 and M2 macrophage polarization stimuli were not included; see text for details.

[2] Abbreviations: NS, healthy nonsmokers; S, healthy smokers; N.S., non-significant.

[3] "M profile" of healthy smokers *vs* healthy nonsmokers, COPD smokers *vs* nonsmokers, and COPD smokers *vs* healthy smokers are indicated (↑ = up-regulated; - = no change; ↓ = down-regulated, $p<0.05$).

* probe sets with very low expression level (average P call < 20%)

** fold change in expression between indicated groups can not be determined due to very low or no expression