

A Distinctive Alveolar Macrophage Activation State Induced by Cigarette Smoking

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Rationale: Macrophages are believed to play a central role in emphysema based largely on data from mouse models. However, the relevance of these models to smoking-related lung disease in humans is uncertain.

Objectives: We sought to comprehensively characterize the effects of smoking on gene expression in human alveolar macrophages and to compare these with effects seen in transgenic mouse models of emphysema.

Methods: We used DNA microarrays with genomewide coverage to analyze alveolar macrophages from 15 smokers, 15 nonsmokers, and 15 subjects with asthma (disease control). Selected gene expression changes were validated by polymerase chain reaction and ELISA. Expression changes were compared with those identified by microarray analysis of interleukin-13-overexpressing and integrin- β 6-deficient mice, which both develop emphysema.

Measurements and Main Results: All 15 smokers shared a common pattern of macrophage gene expression that distinguished them from nonsmokers, a finding not observed in subjects with asthma. We identified 110 genes as differentially expressed in smokers despite using conservative statistical methods. Matrix metalloproteinase 12, a proteinase that plays a critical role in mouse models, was the third most highly induced gene in smokers (ninefold, $p < 0.0001$). However, most changes in smokers were not reflected in mouse models. One such finding was increased osteopontin expression in smokers (fourfold, $p = 0.006$), which was confirmed at the protein level and correlated with the degree of airway obstruction.

Conclusions: Smoking induces a remarkably consistent and distinctive pattern of alveolar macrophage activation. These studies identify aspects of mouse models that are directly relevant to human smokers and also reveal novel potential mediators of smoking-related diseases.

Keywords: gene expression profiling; matrix metalloproteinases; osteopontin; pulmonary emphysema

There are an estimated 1.3 billion habitual cigarette smokers worldwide (1) and 46 million adult smokers in the United States (2). Smoking-related lung diseases include chronic obstructive pulmonary disease (COPD), which comprises emphysema, chronic

bronchitis, and small airway disease (3), as well as several interstitial lung diseases (4). Cigarette smoke induces inflammation, oxidative stress, and tissue injury in the respiratory tract (5). Among the inflammatory changes observed in smokers are changes in the number (6) and function (7, 8) of alveolar macrophages.

How human alveolar macrophages may contribute to the development of emphysema in habitual smokers is a topic of ongoing investigation. The expression and/or function of several proteinases, including matrix metalloproteinase 1 (MMP-1), MMP-2, MMP-9, and MMP-14 (9–15) and cathepsins L and C (16–18), have been found to be increased in studies of smokers and subjects with COPD, and polymorphisms in the *MMP1* and *MMP12* genes have been associated with decline in lung function in smokers (19). These data suggest that macrophages may contribute to emphysema through production of these proteinases.

Activated alveolar macrophages clearly contribute to the development of emphysema in several mouse models, including those with chronic exposure to cigarette smoke (20), airway overexpression of the cytokine interleukin 13 (IL-13) (21), and deletion of an epithelial integrin subunit (β 6) required for transforming growth factor β 1 (TGF- β 1) activation in the lung (22). In each of these models, lung macrophage activation plays a critical role through the production of MMP-12 and other proteinases that contribute to the destruction of alveolar walls (20–22). Although MMP-12 is the proteinase that is most strongly implicated in mouse models of emphysema, studies of MMP-12 expression in humans with COPD have produced inconsistent results (10, 13, 23, 24). These studies have yielded some uncertainty as to the role of MMP-12 in human emphysema and how the products of activated alveolar macrophages may contribute to smoking-related lung disease in humans (25).

We hypothesized that habitual cigarette smoking causes a reproducible and characteristic pattern of macrophage activation in humans. We also hypothesized that the macrophage activation state in human smokers would be similar to that seen in transgenic mouse models of emphysema. To test these hypotheses, we performed genomewide mRNA expression analysis of alveolar macrophages from smokers and control subjects using DNA microarrays. Our results demonstrate that human smokers have a strikingly consistent pattern of macrophage activation. This form of macrophage activation was not seen in healthy nonsmokers or in subjects with asthma, a different cause of airway inflammation and obstructive lung disease. There were some notable similarities to results obtained in two different transgenic mouse models, such as the increase in MMP-12 expression seen in all smokers and in both mouse models. However, most of the macrophage gene expression changes seen in smokers were not seen in the mouse models. The consequences of some of these changes were detectable at the protein level, correlated with lung function in smokers, and are likely to have important effects on inflammation and the extracellular matrix. Some of the results of these studies have been reported in abstract form (26).

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METHODS

Human Subjects

The University of California, San Francisco (UCSF) Committee on Human Research approved this study. Signed informed consent was obtained from all subjects. We studied 15 current cigarette smokers, 15 healthy nonsmoking control subjects, and 15 nonsmoking subjects with asthma. Spirometry, methacholine challenge testing, measurement of diffusing capacity (smokers only), and bronchoscopy with bronchoalveolar lavage (BAL) were performed as described previously (27). Macrophages were isolated by flow cytometry using forward scatter and autofluorescence characteristics (28). Macrophage purity was $98 \pm 2\%$ by Diffquik staining, with no difference between groups. Additional information about inclusion and exclusion criteria, study design, and procedures is provided in an online supplement.

Mice

Animal studies were approved by the UCSF Institutional Animal Care and Use Committee. Integrin- $\beta 6$ -deficient (*Itgb6*^{-/-}) mice (29) on an FVB background were generously provided by Dean Sheppard (University of California, San Francisco). These mice spontaneously develop progressive airspace enlargement over a period of months. Transgenic mice with CC10 promoter-driven over-expression of IL-13 (30) were generously provided by Jack Elias (Yale University, New Haven, CT). Mice used for these experiments were *Tg(CC10-IL-13)Stat6*^{+/-} mice on a Balb/c background (herein referred to as IL-13 tg) (31). These mice (like *Tg[CC10-IL-13]Stat6*^{+/+} mice) develop spontaneous airspace enlargement by 2 mo of age (31). Macrophages were collected from 2-mo-old *Itgb6*^{-/-} mice, IL-13 tg mice, and littermate control mice by BAL. There were three mice in each group. In addition, macrophages were collected from three 10-mo-old *Itgb6*^{-/-} mice to determine the effect of age and progression of emphysema on macrophage gene expression. As previously reported (22, 31), we found increased proportions of inflammatory cells other than macrophages in BAL fluid (BALF) from IL-13 tg mice (18% lymphocytes, 37% granulocytes) and *Itgb6*^{-/-} mice (29% lymphocytes and < 1% granulocytes at 2 mo, 24% lymphocytes and < 1% granulocytes at 10 mo). To purify macrophages, BALF cells were plated for 30 min at 37°C and nonadherent cells were removed. Of adherent cells used for microarray analysis, more than 97% were macrophages as shown by Diffquik staining for all experimental groups.

Analysis of Alveolar Macrophage RNA

RNA was extracted from mouse and human alveolar macrophages and DNase-treated using the RNeasy kit (Qiagen, Valencia, CA). RNA concentration and quality were assessed using the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA).

For mouse microarray studies, total RNA (250 ng/mouse) was amplified using one round of *in vitro* transcription with incorporation of biotinylated nucleotides (Message Amp II aRNA kit no. 1751; Ambion, Austin, TX). Amplified cRNA samples were hybridized to Mouse Genome 430 2.0 microarrays (Affymetrix, Santa Clara, CA) with each mouse sample run on a separate array. For human samples, smaller quantities of RNA (50 ng) were available. Therefore, RNA was amplified by performing two rounds of *in vitro* transcription (Ambion Mes-

sage Amp aRNA kit no. 1750). Samples were hybridized to Affymetrix Human Genome U133 Plus 2.0 GeneChips.

Microarray Data Analysis

Array images were analyzed using Affymetrix GeneChip Expression Analysis software. Bioconductor (32) was used for quality control (affyPLM algorithm), preprocessing (Robust Multichip Average algorithm), cluster analysis, and linear modeling (32–34). Hierarchic clustering was performed using Pearson correlation. Differential gene expression was assessed using linear models (controlling for age and sex in human samples). For each probe set, we computed the fold change and p value using the Bonferroni adjustment for multiple comparisons. We considered Bonferroni-adjusted p values less than 0.05 as statistically significant. This provides a family-wise type I error rate of less than 5%, meaning that there was only a 5% chance of falsely identifying any differentially expressed genes if the null hypothesis were correct (e.g., if smoking has no effect on gene expression) (35). Gene expression levels were correlated with measures of lung function using Pearson correlation. Array data are available from the Gene Expression Omnibus public database (<http://www.ncbi.nlm.nih.gov/geo/>, accession number GSE2125).

Quantitative Real-Time Polymerase Chain Reaction

Transcript copy number for specific genes of interest was measured using an adaptation of a two-step real-time reverse transcriptase-polymerase chain reaction (PCR) method described previously (36) and in the online supplement.

Protein Assays

BALF was concentrated fivefold (human samples) or 3.5-fold (mouse samples) using Microcon filters (Millipore, Bedford, MA). Osteopontin and insulinlike growth factor-1 (IGF-1) levels were measured using Quantikine kits (R&D Systems, Minneapolis, MN).

RESULTS

Analysis of Alveolar Macrophage Gene Expression in Human Smokers and Subjects with Asthma

We studied alveolar macrophages from 15 habitual cigarette smokers, 15 nonsmoking healthy control subjects, and 15 nonsmoking subjects with asthma. We included the asthmatic group of subjects to assess whether the changes induced by smoking are distinctive or reflect nonspecific airway inflammation. Although all subjects were recruited from the same local population with standardized inclusion criteria, healthy control subjects differed from smokers with respect to age and sex (Table 1). Both smokers and subjects with asthma had lower lung function than healthy control subjects. Smokers were all currently smoking cigarettes. Smokers were using 1.4 ± 0.7 packs/d (mean \pm SD) at the time of enrollment and lifetime smoking exposure was 47 ± 27 pack-yr. Two smokers had mild COPD (as defined by Global Initiative for Chronic Lung Disease [GOLD] class 1), and five subjects had moderate COPD (GOLD class 2). Five

TABLE 1. SUBJECT CHARACTERISTICS

	Control Subjects	Smokers	Subjects with Asthma
No. subjects	15	15	15
Sex, F/M	10/5	2/13*	9/6
Age, yr	41 \pm 8	51 \pm 8*	35 \pm 10
FEV ₁ prebronchodilator, % predicted	104 \pm 12%	80 \pm 15%*	81 \pm 15%*
FEV ₁ /FVC prebronchodilator	0.80 \pm 0.06	0.62 \pm 0.12*	0.70 \pm 0.11*
FEV ₁ post-bronchodilator, % predicted	107 \pm 12%	84 \pm 16%*	91 \pm 12%*
FEV ₁ /FVC post-bronchodilator	0.83 \pm 0.05	0.67 \pm 0.14*	0.77 \pm 0.09
PC ₂₀ , mg/dl methacholine	64 (64, 64)	11 (1.6, 48)*	0.5 (0.06, 1.2)*

Definition of abbreviations: F = females; M = males; PC₂₀ = the concentration of methacholine that causes a 20% decline in FEV₁. Data are presented as mean \pm SD or median (interquartile range).

* p < 0.05 compared with nonsmoking healthy control subjects.

smokers had mild reductions in diffusing capacity for carbon monoxide (60–80% of predicted), suggestive of mild emphysema.

Macrophage RNA was obtained from subjects in all three groups using a standardized approach and analyzed on microarrays containing 54,675 probe sets. We first used hierarchic clustering to group macrophage samples based on the pattern of expression of the 200 most variable genes. These variable genes were selected from the entire set of 30 subjects, without regard to whether they were differentially expressed in the 15 smokers as compared with the 15 nonsmokers. Nonetheless, we found that samples from all 15 smokers grouped together in one cluster, whereas samples from all 15 nonsmokers grouped together in a separate cluster (Figure 1A). Similar results were obtained when larger numbers of genes were used for clustering: there was complete segregation of smokers from control subjects when the 300 most variable genes were used, and only one smoker clustered with the control subjects when the 500 or 1,000 most variable genes were used (data not shown). This indicates that

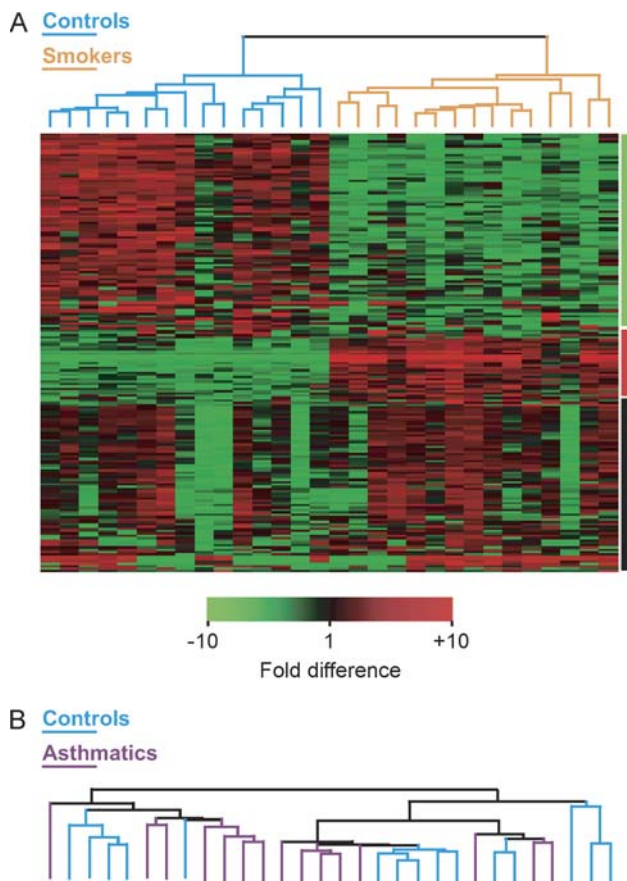


Figure 1. Cigarette smoking had a robust and consistent effect on gene expression in human alveolar macrophages that was not observed in subjects with stable asthma. Macrophage RNA from smokers, control subjects, and subjects with asthma was analyzed on DNA microarrays. (A) Cluster analysis using data for the 200 probe sets with the highest variance across 15 smokers and 15 nonsmoking control subjects completely segregated the smokers from control subjects. The *color map* depicts fold differences in intensity as compared with the median value for all subjects. *Colored bars* at right indicate genes with increased expression in smokers (*red*), decreased expression in smokers (*green*), and genes unrelated to smoking status (*black*). (B) The same approach did not distinguish macrophage gene expression in 15 nonsmoking subjects with asthma from that in 15 nonsmoking control subjects without asthma (as indicated by *dendrogram*).

cigarette smoking had robust and consistent effects on gene expression in human alveolar macrophages. By contrast, when we performed the same analyses using alveolar macrophages from 15 subjects with asthma and the same healthy control subjects, we found that hierarchic clustering did not segregate asthmatic samples from healthy nonsmoking control samples (Figure 1B).

We next used linear models that controlled for age and sex to identify statistically significant gene expression differences between smokers and healthy nonsmoking control subjects. Using the conservative Bonferroni correction for multiple comparisons, we found differences with 123 probe sets representing 103 named genes (some with duplicate probe sets) and 7 unnamed genes (Table E1 in the online supplement). Seventy-two of these genes were increased and 38 were decreased in smokers compared with healthy nonsmoking control subjects. The gene most highly induced in smokers (Table 2) was CYP1B1 (99-fold induced by array), a cytochrome P450 enzyme that is known to be induced by cigarette smoke (37). The gene that ranked third for fold induction was MMP-12 (ninefold), the macrophage-specific MMP implicated in several mouse models of emphysema (20–22). When we used linear models to identify statistically significant gene expression changes in macrophages from subjects with asthma, we found that only 10 genes were differentially expressed between subjects with stable asthma and healthy nonsmokers, and none of these 10 were among the genes that were differentially expressed in smokers (Table E2). The expression of 12 genes found to be differentially expressed in alveolar macrophages from smokers was further evaluated by quantitative real-time PCR. Differential expression was confirmed for all 12 transcripts, including MMP-12 (Figure 2). In some cases, the fold changes determined by PCR were larger than those estimated using microarrays, probably due to the greater sensitivity and dynamic range of PCR.

Analysis of Alveolar Macrophage Gene Expression in Two Mouse Models of Emphysema

To determine whether macrophage activation in mouse models of emphysema mirrors macrophage activation observed in human smokers, we analyzed alveolar macrophages from two transgenic mouse lines that develop emphysema, IL-13-overexpressing mice and *Itgb6*^{-/-} mice, using DNA microarrays containing 45,101 probe sets. We studied IL-13-overexpressing mice at 2 mo of age because previous studies indicate that these mice have emphysema by this age (31). We studied *Itgb6*^{-/-} mice at 2 mo of age, when MMP-12 expression is maximal but emphysema is not yet detectable (22), and also at 10 mo, when there is obvious emphysema (Figure 3 and Reference 22). We again began data analysis by using a hierarchic clustering approach to identify dominant patterns of gene expression in these two groups of mice and the two littermate control groups (Figure 4). The expression of a minority of genes depended principally on the genetic background of the mice (Balb/c vs. FVB). However, most changes related to genes that were increased or decreased in experimental versus control mice. In these analyses, IL-13-overexpressing and *Itgb6*^{-/-} mice clustered together rather than with their littermate control animals. The pattern of gene expression in macrophages from 2- and 10-mo-old *Itgb6*^{-/-} mice was remarkably similar (Figure 4). These results demonstrate that alveolar macrophages from these two mouse models of emphysema manifest very similar patterns of gene expression changes.

We found a large number of differentially expressed genes in macrophages from IL-13-overexpressing or *Itgb6*^{-/-} mice as compared with their littermate control animals. In IL-13-overexpressing mice, significant differential expression (Bonferroni adjusted $p < 0.05$) was detected with 2,037 probe sets representing

TABLE 2. THE 10 MOST HIGHLY INDUCED AND REPRESSED GENES IN ALVEOLAR MACROPHAGES FROM SMOKERS

Probe Set Identifier	Symbol	Description	Fold Difference	Adjusted p Value
Increased Expression				
202437_s_at	CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1	99.0	2.8×10^{-12}
206214_at	PLA2G7	Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)	19.4	7.0×10^{-10}
204580_at	MMP12	Matrix metalloproteinase 12	8.8	2.5×10^{-5}
212158_at	SDC2	Syndecan 2	6.1	2.2×10^{-3}
223204_at	DKFZp434L142	Hypothetical protein	5.1	1.1×10^{-4}
223503_at	DKFZp566N034	Hypothetical protein	5.0	2.7×10^{-4}
228285_at	TDRD9	Tudor domain containing 9	4.8	1.2×10^{-5}
240137_at	TDRD6	Tudor domain containing 6	4.8	1.2×10^{-4}
203178_at	GATM	Glycine amidinotransferase	4.5	3.8×10^{-2}
219648_at	DSU	Likely ortholog of mouse dilute suppressor	4.5	1.4×10^{-5}
Decreased Expression				
227399_at	FLJ38507	Colon carcinoma-related protein	-13.1	1.5×10^{-6}
219304_s_at	PDGFD	Platelet-derived growth factor D	-11.4	4.9×10^{-6}
209795_at	CD69	CD69 antigen	-8.5	1.1×10^{-3}
219064_at	ITIH5	Inter- α inhibitor H5	-7.5	1.3×10^{-7}
239345_at	SLC19A3	Solute carrier family 19, member 3	-6.7	2.0×10^{-3}
242836_at	—	—	-5.4	3.9×10^{-2}
209541_at	IGF1	Insulinlike growth factor 1	-4.9	2.9×10^{-2}
203917_at	CXADR	Coxsackie virus and adenovirus receptor	-4.5	2.8×10^{-2}
207111_at	EMR1	Egf-like module containing mucin-like, hormone receptor-like 1	-4.3	1.6×10^{-3}
228608_at	VGCNL1	Voltage-gated channel-like 1	-4.2	2.9×10^{-6}

approximately 1,098 genes (Table E3). In 2-mo-old *Itgb6*^{-/-} mice, differential expression was detected with 2,371 probe sets representing approximately 1,215 genes (Table E4). Many genes with known roles in extracellular matrix remodeling and regulating inflammation had altered expression in macrophages from both models (Table 3). These included proteinases and protein-

ase inhibitors, chemokines, and complement components. A smaller number of genes stood out as highly differentially expressed in only one of the two models. For example, arginase 1, a well-recognized marker of the alternative macrophage activation state ascribed to T-helper type 2 cytokine stimulation in other systems (38, 39), was the second most highly induced gene in the IL-13-overexpressing model (370-fold increase; Table E3) but was not significantly induced in *Itgb6*^{-/-} mice. Other genes previously reported to be characteristic of alternative macrophage activation (40–43) that were also exclusively or disproportionately elevated in the IL-13 overexpression model included resistin-like α /FIZZ1 (increased 51-fold in IL-13-overexpressing vs. 3.7-fold in *Itgb6*^{-/-} mice), eosinophil-associated RNase A family member 11 (155-fold vs. 2.8-fold increase), chitinase 3-like 4/Ym2 (6.5-fold increase vs. 4.2-fold decrease), and fibronectin (8.5- vs. 2.6-fold increase). Genes that were highly induced in *Itgb6*^{-/-} mice but were not significantly induced in IL-13-overexpressing mice included the complement receptor C1qr1 (96-fold), maltase-glucoamylase (77-fold), interferon-induced transmembrane protein 6 (42-fold), and interferon-activated gene 205 (24-fold).

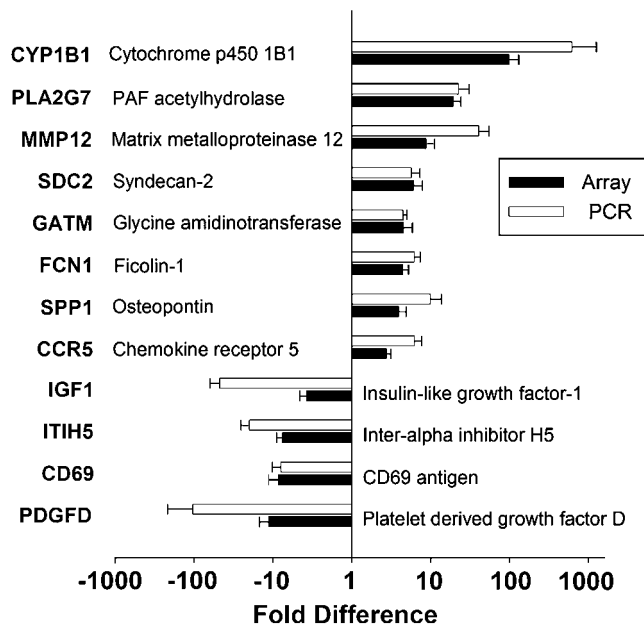


Figure 2. Quantitative polymerase chain reaction (PCR) validation of differential gene expression detected using microarrays. Fold differences as determined by microarray and real-time PCR in macrophages from smokers as compared with healthy control subjects are presented for 12 genes found to be differentially expressed by microarray. For all 12 genes, real-time PCR data confirmed statistically significant differential expression in smokers (adjusted $p < 0.05$). PAF = platelet-activating factor.

Comparison of Humans and Mouse Models

To determine whether the gene expression changes in alveolar macrophages from smokers mirror those observed in mouse models of emphysema, we identified mouse orthologs for 81 of the genes differentially expressed in smokers by microarray analysis. For 51 of these 81 genes (63%), we did not detect significant changes in expression of mouse orthologs in either mouse model of emphysema at 2 mo of age. For 17 of these 81 genes (21%), concordant expression changes were observed in smokers and one or both of the mouse models (Table 4). For 13 genes (16%), the expression changes were discordant (in the opposite direction) in smokers and one or both mouse models (Table 4). Substantial differences in macrophage activation between human cigarette smokers and the two mouse emphysema models that we studied at 2 mo of age are apparent when the largest gene expression changes in macrophages from smokers

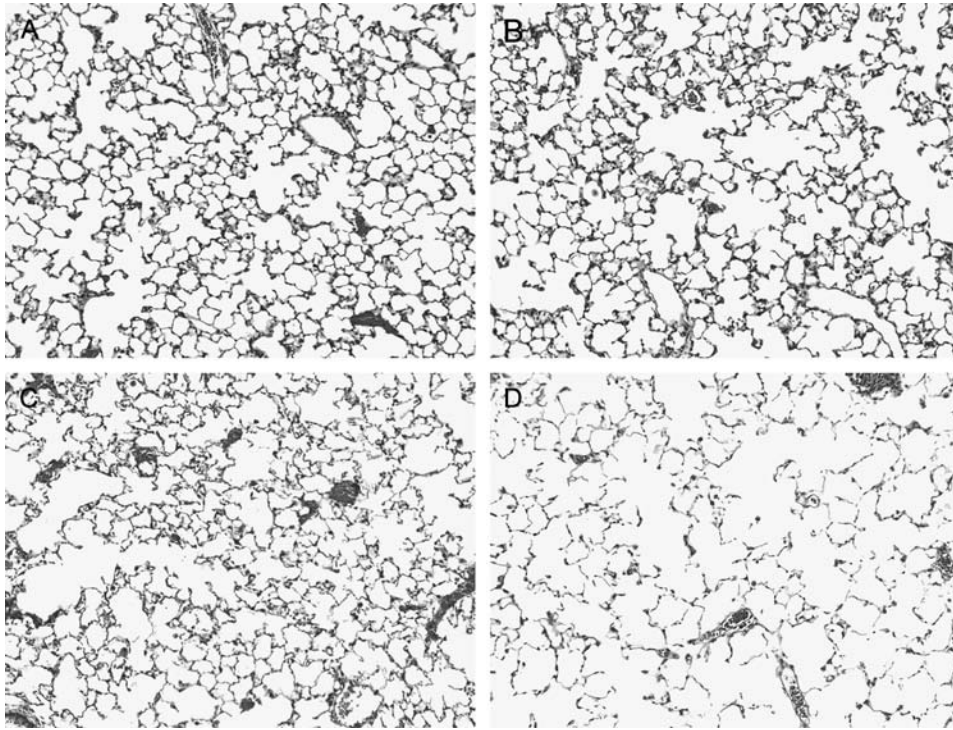


Figure 3. Histologic appearance of lungs from 2-, 6-, and 10-mo-old integrin- β 6-deficient mice. Sections of lung of 2-mo-old wild-type control mice (A) and 2-mo-old (B), 6-mo-old (C), and 10-mo-old (D) integrin- β 6-deficient mice were stained with hematoxylin and eosin and photographed using a 10 \times objective.

are compared with mouse model results for orthologous genes (Figure 5). We also examined the expression of orthologous genes in *Itgb6*^{-/-} mice at 10 mo of age, when the pathologic changes of emphysema are more evident. We found that 10- and 2-mo-old *Itgb6*^{-/-} mice were very similar in the expression of the 81 mouse orthologs of genes that were altered in human smokers. These analyses yielded only one additional gene that overlapped with the expression changes found in human smokers (Table E6). This gene, phosphodiesterase 4B (PDE4B), was increased in smokers and in 10-mo-old β 6-deficient mice. There was a trend toward increased expression in 2-mo-old *Itgb6*^{-/-} mice compared with 2-mo-old littermate control animals, but this did not reach statistical significance after Bonferroni adjustment. Thus, studying the disease process at a more advanced stage did not substantially affect the degree of overlap between the mouse model and human smokers.

Protein Verification

The two most highly differentially expressed transcripts in smokers that give discordant results in the mouse models encode secreted proteins, IGF-1 and osteopontin (Table 4). To determine whether the observed changes in RNA transcript levels were associated with differences in levels of the corresponding proteins in lung extracellular fluid, we used ELISA to measure concentrations of osteopontin and IGF-1 proteins in BALF. Macrophage IGF-1 mRNA expression was increased in both mouse models (Table 4), and these changes were mirrored by increases in BALF IGF-1 protein in both IL-13-overexpressing mice ($2,160 \pm 560$ vs. 160 ± 20 pg/ml in strain-matched control animals, $p = 0.007$) and *Itgb6*^{-/-} mice (280 ± 30 vs. 140 ± 10 pg/ml in control animals, $p = 0.002$). In contrast, IGF-1 macrophage mRNA was decreased in smokers and there was a trend toward lower IGF-1 protein expression in BALF from smokers as compared with control subjects (40 ± 40 vs. 260 ± 140 pg/ml, $p = 0.15$). Macrophage osteopontin mRNA expression was decreased in *Itgb6*^{-/-} mice and unchanged in IL-13-overexpressing mice (Table 4). The change in *Itgb6*^{-/-} mice was mirrored by

decreased BALF osteopontin protein levels (Figure 6A). In contrast, osteopontin mRNA and protein were both increased in smokers (Table 4 and Figure 6B). These data indicate that specific differences in IGF-1 and osteopontin transcript expression seen in human smokers and mouse models are associated with similar changes in levels of the corresponding proteins in lung epithelial lining fluid.

Gene Expression and Lung Function

Because osteopontin transcript and protein levels were markedly increased in smokers and osteopontin is known to regulate lung inflammatory responses, we explored the possibility that osteopontin expression might be associated with impaired lung function in smokers. We found that increased osteopontin expression correlated with decreased lung function within the group of smokers as measured by post-bronchodilator FEV₁/FVC (Figure 6C) or FEV₁ (not shown). No such relationship was apparent for other differentially expressed genes. For example, MMP-12 expression was elevated in all smokers as compared with healthy control subjects and MMP-12 levels did not correlate with lung function in the 15 smokers (Figure 6D).

DISCUSSION

We used an integrative genomics approach to define the characteristics of macrophage activation in human smokers and in two transgenic mouse models of emphysema. We found that cigarette smoking had a remarkably potent and consistent effect on macrophage activation in human subjects, a finding that was not observed in subjects with asthma. Among the most highly induced genes in human smokers was MMP-12, a proteinase believed to be important in the pathogenesis of emphysema on the basis of mouse models. However, many of the gene expression changes seen in smokers would not have been predicted from analyses of the two mouse models of emphysema. For example, we found that osteopontin was highly induced in macrophages from smokers but not in the mouse models, and that increased

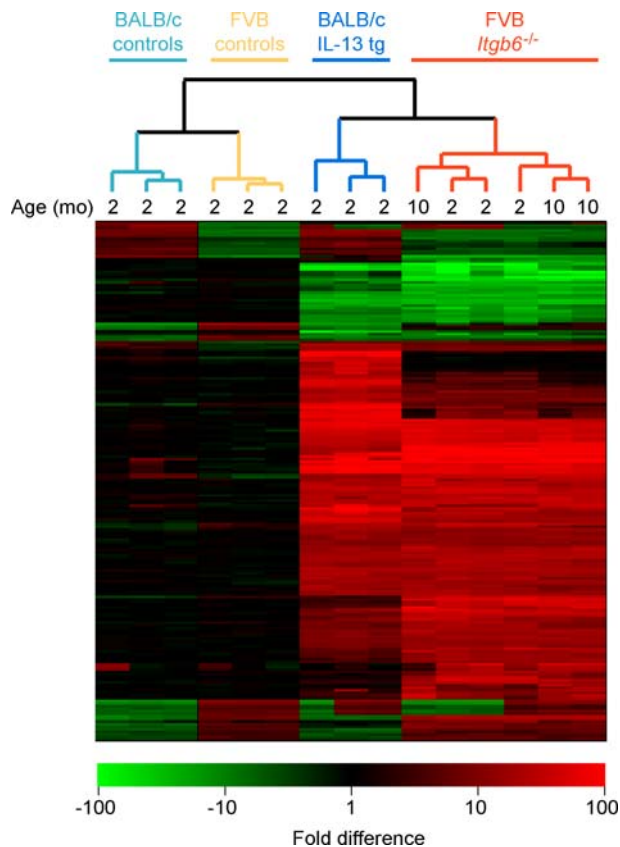


Figure 4. Similar patterns of gene expression in alveolar macrophages from interleukin-13–overexpressing (IL-13 tg) and integrin- β 6–deficient (*Itgb6*^{-/-}) mice. Macrophage RNA from 2-mo-old IL-13 tg and *Itgb6*^{-/-} mice and their respective wild-type control animals (BALB/c and FVB) were analyzed on DNA microarrays (n = 3/group). Additional *Itgb6*^{-/-} mice were studied at 10 mo of age. Samples and genes were clustered using expression data from the 200 probe sets with the highest variance. The *color map* indicates fold difference as compared with the median value for all six control animals (1 = no difference).

expression levels correlated with lung function impairment in smokers. These analyses indicate that habitual cigarette smoking has a distinctive and reproducible effect on macrophage activation and identify specific genes that may contribute to the pathogenesis of COPD.

We found that the effects of cigarette smoking on alveolar macrophage gene expression were reproducible and distinctive despite a very conservative approach to data analysis. We performed hierarchic clustering of samples according to expression of the most variable genes to determine the predominant sources of variation in gene expression in an unbiased manner (without using information about smoking status). We found that all 15 smokers completely segregated from all 15 nonsmoking control subjects (Figure 1A), but that the 15 subjects with asthma could not be distinguished from control subjects (Figure 1B). This indicates that smoking status accounted for much of the observed variation in gene expression and that smoking induces a dramatic and highly consistent change in macrophage gene expression patterns. To identify differentially expressed genes, we applied the conservative Bonferroni approach to adjust for the very large number of probe sets on the microarrays. Even using this conservative approach, we identified 110 genes as differentially expressed in smokers versus nonsmoking control subjects. By contrast, only 10 genes were differentially expressed in macrophages from subjects with asthma, and none of these genes overlapped with findings in smokers. Quantitative PCR confirmed differential expression of genes in smokers identified by microarray. There are likely to be additional genes induced by smoking that were not identified in microarray analyses due to our conservative approach to data analysis and to the limitations of microarrays for detection of low abundance transcripts. In support of this idea, we used real-time PCR to analyze expression of proteinases not identified as differentially expressed in array studies and found evidence that some of these (MMP-2, MMP-9, and cathepsin K) were increased in smokers (data not shown). Although microarray-based studies have certain intrinsic limitations in sensitivity, we were still able to find clear and convincing evidence for extensive smoking-induced changes in macrophage gene expression using this approach.

We compared macrophage gene expression changes in human smokers with those seen in two transgenic mouse models of emphysema to identify common features that might be involved in emphysema pathogenesis. Previous work suggests that development of emphysema in these two mouse models is driven by very different mechanisms. IL-13–overexpressing mice develop emphysema very rapidly and have granulocytic inflammation and increased TGF- β levels (44), whereas *Itgb6*^{-/-} mice develop emphysema more slowly, do not have granulocytic inflammation, and are deficient in functional TGF- β (22). Given these differences, we were surprised to find that the pattern of macrophage activation was strikingly similar in these two models (Figure 4 and Table 3). In contrast, the degree of similarity between human

TABLE 3. SELECTED GENES DIFFERENTIALLY EXPRESSED IN BOTH INTERLEUKIN-13–OVEREXPRESSING AND INTEGRIN- β 6–DEFICIENT MICE

Gene Category	Increased	Decreased
Matrix metalloproteinases	Mmp12 , Mmp13 , Mmp19	—
Cathepsins	Ctsb, Ctse, Ctsk, Ctsl	—
Other proteinases	Adam8 , Adam19, urokinase plasminogen activator	Adam22
Serine (or cysteine) proteinase inhibitors	Serpib6b, Serpinb8	Serpib1a
Tissue inhibitors of metalloproteinase	Timp1 , Timp2	—
Other proteinase inhibitors	Cystatin B, Expi	Kazald1
Matrix components	Fibronectin 1, fibrinogen-like protein 2 , thrombospondin 1	—
Chemokines	Ccl9, Ccl12, Ccl17, Ccl22, Cxcl4, Cxcl16	—
Complement components	C1qa , C1qb , C1qg , Cfh	—

Genes listed were differentially expressed in both mouse lines compared with appropriate control animals (p < 0.05 after Bonferroni correction). Genes listed in boldface type were changed by more than 10-fold in at least one of the two mouse models.

TABLE 4. GENES DIFFERENTIALLY EXPRESSED IN HUMAN SMOKERS AND AT LEAST ONE MOUSE MODEL OF EMPHYSEMA

Symbol	Description	Fold Difference		
		Smokers	IL-13 tg	<i>Itgb6</i> ^{-/-}
Concordant Changes				
PLA2G7	PAF acetylhydrolase	19.4	3.8	3.0
MMP12	Matrix metalloproteinase 12	8.8	14.5	22.1
DKFZp434L142	Hypothetical protein	5.1	22.6	35.0
DSU	Likely ortholog of mouse dilute suppressor	4.5	5.2	4.3
CLECSF5	C-type lectin, superfamily member 5	4.3	4.1	4.4
BASP1	Brain-abundant, membrane-attached signal protein 1	3.3	3.0	2.8
FAM20C	Family with sequence similarity 20, member C	3.3	6.2	4.5
TREM2	Triggering receptor expressed on myeloid cells 2	3.1	5.3	4.5
SLC26A11	Solute carrier family 26, member 11	2.9	1.8	—
CCR5	Chemokine (C-C motif) receptor 5	2.7	15.8	7.7
SLC7A11	Solute carrier family 7, member 11	2.5	3.1	—
CCL2	Chemokine (C-C motif) ligand 2	1.9	6.2	11.1
CSTB	Cystatin B	1.5	1.7	1.5
C10orf128	Hypothetical protein	-1.7	—	-2.6
NFIA	Nuclear factor I/A	-2.3	—	-1.7
RND3	Rho family GTPase 3	-3.9	-7.8	—
RGS2	Regulator of G-protein signaling 2	-3.9	-3.0	—
Discordant Changes				
SPP1	Osteopontin	4.0	—	-13.9
FLT1	Vascular endothelial growth factor receptor	2.5	—	-24.3
TGFBR1	Transforming growth factor, β receptor I (activin A receptor type II-like kinase, 53 kD)	2.2	-1.8	-2.5
GSR	Glutathione reductase	2.1	—	-4.1
WWTR1	WW domain containing transcription regulator 1	1.7	-6.2	-24.3
FGR	Gardner-Rasheed feline sarcoma viral oncogene homolog	1.6	—	-2.1
MGC15619	Hypothetical protein	1.5	—	-2.8
RAB11FIP5	RAB11 family interacting protein 5 (class I)	-1.6	—	1.7
C2	Complement component 2	-1.7	2.4	—
ITSN1	Intersectin 1	-2.5	3.1	1.9
THBS1	Thrombospondin 1	-3.6	56.3	38.6
NR4A2	Nuclear receptor subfamily 4, group A, member 2	-3.7	—	6.5
IGF1	Insulinlike growth factor 1	-4.9	89.4	6.4

Definition of abbreviation: IL = interleukin; PAF = platelet-activating factor.

All values represent statistically significant differences compared with appropriate controls (Bonferroni adjusted $p < 0.05$). Dashes (—) indicate no significant difference.

smokers and the mouse models was much more restricted (Table 4 and Figure 5). There are inevitable limitations inherent in comparing expression microarray data between species. Different microarrays must be used for human and mouse samples, and it is not always possible to unambiguously identify mouse orthologs of human genes. Furthermore, our between-species comparison may be affected by technical differences in the way that mouse and human macrophages were collected and purified and in the methods used to amplify mouse and human macrophage mRNAs. Nonetheless, our finding that there were almost as many discordant changes as concordant changes shows that there are major differences between the human and mouse macrophage activation states. On the one hand, this finding focuses attention on a relatively small number of genes that could have a role in emphysema pathogenesis in both human smokers and in mouse models of emphysema. On the other hand, the finding helps to identify unique features of smoking-induced macrophage activation that may not be modeled by these transgenic systems.

One of the genes induced both in human smokers and in mouse models was the proteinase MMP-12. Using arrays with more than 50,000 probe sets, MMP-12 was identified as the third most highly induced gene in alveolar macrophages from smokers, and we confirmed previous reports that MMP-12 is highly induced in each of the mouse models (21, 22). MMP-12 is required for the development of emphysema in *Itgb6*^{-/-} mice and in

cigarette smoke-exposed mice (20, 22). However, whether MMP-12 contributes to human emphysema has been uncertain, due in part to conflicting evidence about whether this proteinase is induced in human smokers (10, 13, 23, 24). Using quantitative PCR, which has a very large, dynamic range, we measured an even greater degree of induction of MMP-12 transcripts in alveolar macrophages from smokers than estimated by the arrays (41-fold; Figure 2). However, we, like some others (10, 13), were unable to reliably detect MMP-12 protein or activity by casein zymography, Western blotting, or fluorokine multi-analyte profiling assays with antibody-coated microparticles performed on BALF concentrated using Microcon filters (data not shown). In a recent report (23), MMP-12 protein was detected in highly concentrated lyophilized BALF from subjects with COPD. Thus, we are uncertain whether the large and consistent smoking-induced increases in MMP-12 transcript levels that we observed lead to increased MMP-12 protein expression or function. In addition, our data suggest that increased MMP-12 expression may not in itself be sufficient to cause emphysema because expression was increased in all smokers independent of the degree of impairment in lung function (Figure 6D). Other genes induced in both human smokers and in mouse models include platelet-activating factor acetylhydrolase (PLA2G7, the second most highly induced gene in smokers), monocyte chemoattractant protein 1 (CCL2), and chemokine receptor 5 (CCR5; Table 4). Mouse models will likely continue to be useful tools for helping

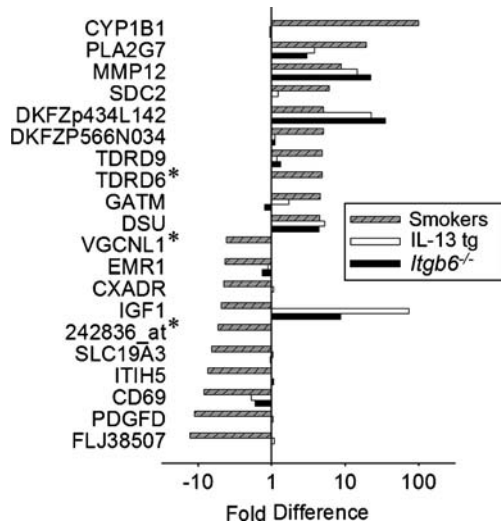


Figure 5. The most highly induced and repressed genes in alveolar macrophages from smokers compared with results for orthologous genes from mouse models. The 10 genes with greatest increase and decrease in expression in smokers are presented with shaded, hatched bars indicating fold difference as compared with controls. Open bars denote fold difference for the orthologous mouse gene in IL-13-overexpressing mice as compared with wild-type BALB/c controls, and black bars denote fold difference for $\beta 6$ -deficient mice as compared with FVB control animals. For three human genes (TDRD6, VGCNL1, and 242836_at; denoted by asterisks), no orthologous gene was represented on the mouse arrays.

to investigate the possible contributions of these genes to emphysema.

Many of the gene expression changes induced by smoking were not apparent in either of the transgenic mouse models. A notable example is the enzyme CYP1B1, the most highly induced gene in smokers. This cytochrome P450-family protein is known to be induced by cigarette smoke (37, 45) and produce carcinogenic DNA adducts in the process of detoxifying combustion products in smoke. Another gene induced only in smokers was glutathione reductase (2.1-fold induced, adjusted $p < 0.01$), which plays an important role in protection against oxidative stress in human alveolar macrophages (46). We found that IGF-1 transcripts were decreased in smokers but increased in mouse models and confirmed these changes at the protein level in BALF. IGF-1 inhibits apoptosis of lung epithelial cells (47), suggesting that decreases in IGF-1 could contribute to the increased epithelial cell apoptosis seen in human emphysema (48, 49). This pattern of expression also differentiates smoking-induced alveolar macrophage activation from findings in other lung diseases such as idiopathic pulmonary fibrosis, which are characterized by increased macrophage expression of IGF-1 (50–52). Another distinctive gene expression change observed in smokers that contrasted sharply with mouse models was increased expression of osteopontin. BALF protein analysis confirmed increased expression of osteopontin in smokers and decreased expression in $Itgb6^{-/-}$ mice. Osteopontin is a multifunctional protein that has been implicated in macrophage recruitment (53, 54) and plays a role in osteoclast differentiation and activation (55, 56), which suggests parallels between smoke-exposed alveolar macrophages and osteoclasts involved in degrading bone matrix. The role of each of these smoking-induced gene expression changes requires further study in humans and in suitable model systems.

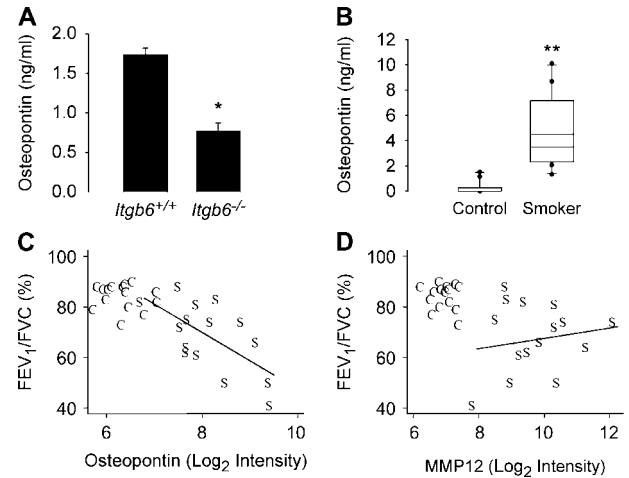


Figure 6. Distinctive changes in bronchoalveolar lavage fluid (BALF) osteopontin protein concentrations in smokers. (A) Osteopontin levels in BALF from control and $Itgb6^{-/-}$ mice; * $p = 0.0001$ versus control animals. (B) Osteopontin levels in BALF from human control nonsmokers and smokers; ** $p = 0.0003$ versus control subjects. (C, D) Relationship of airway obstruction (as measured by post-bronchodilator FEV₁/FVC ratio) to macrophage osteopontin and matrix metalloproteinase (MMP)-12 transcript expression in control nonsmokers (C) and smokers (S). The line indicates the correlation between FEV₁/FVC in smoking subjects and osteopontin or MMP12 expression levels (for osteopontin, $r = -0.64$, $p = 0.011$; for MMP12, $r = 0.17$, $p = 0.55$ by Pearson correlation).

We found that habitual cigarette smoking induces a remarkably consistent pattern of gene expression changes in all of the subjects that we studied, but epidemiologic data suggest that only a subset of smokers develop COPD (57). We speculate that risk and susceptibility factors for COPD could function at three levels. First, the risk of COPD is related to the dose and duration of smoking exposure (58, 59), suggesting that COPD risk may relate to the cumulative effects of the consistently observed changes in macrophage gene expression in smokers. Second, there may be differences between individuals in the response of specific genes to smoking. For example, we found that osteopontin was increased in all smokers, but that the extent of this increase was greater in patients with more severe lung function impairment (Figure 6C). Further studies involving subjects with more extreme lung function abnormalities might allow for the identification of other macrophage gene expression changes that are selectively associated with the development of COPD in a subset of smokers. Third, it seems likely that susceptibility to COPD may relate to differences in the response of the lung to smoking-induced macrophage activation. For example, MMP-12 (which was elevated in smokers without apparent relation to lung function) might produce emphysema more readily in smokers with lower levels of antiproteinase activity. Our findings demonstrate that macrophage function is markedly altered by smoking and provide a useful framework for future studies that focus on contributions of specific macrophage gene expression changes to the pathogenesis of smoking-induced lung disease in humans.

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