COPD Is Associated With a Macrophage Scavenger Receptor-1 Gene Sequence Variation

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Background: Macrophages play an important role in COPD. We genotyped at-risk smokers to evaluate the role of polymorphisms in the macrophage scavenger receptor-1 gene (*MSR1*) in COPD susceptibility and related measures of lung function. Then, in macrophages from donors with specific *MSR1* genotypes, we determined the effect of *MSR1* single nucleotide polymorphisms (SNPs) on macrophage function by examining *in vitro* adhesion, receptor expression, and cell number in culture as an index of increased survival/reduced apoptosis.

Methods: Smokers (≥ 20 pack-years) who were > 40 years ($n = 714$) were genotyped for seven SNPs; one nonsense change (ex6R293 $\times$ _C/T), four missense changes (ex4V113A_T/C, ex4P174Y_G/T, ex11H441R_A/G, and in the ligand binding site ex6P275A_C/G), -176511_A/G in the promoter region, and IVS5-59_C/A in the intron. Nonsmoking healthy volunteers ($n = 85$) were genotyped, and peripheral blood monocytes were isolated from seven P275A_CG/GG and eight P275A_CC controls and cultured to generate monocyte-derived macrophages (MDM). The effectiveness of trypsin and scraping to dislodge MDM was scored on a four-point subjective scale. MDM were counted on a Z1 particle counter and surface expression of *MSR1* was determined by fluorescence-activated cell sorting analysis using secondary staining of antibodies against human macrophage scavenger receptor (*MSR1*).

Results: The *MSR1*-coding SNP P275A was associated with susceptibility to COPD in smokers ($P < .005$) and a lower percent predicted (pp) FEV₁, FEV₁/FVC, and pp forced expiratory flow (FEF)₂₅₋₇₅ ($P = .03$). P275A_CG/GG was also associated with increases in maintenance of cell number in culture (increased survival/reduced apoptosis), *MSR1* expression, and adhesion of macrophages to plastic *in vitro* ($P < .05$).

Conclusions: The *MSR1* association with COPD susceptibility, COPD-related measures of lung function, and abnormalities of macrophage function may account for significant COPD morbidity.

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Abbreviations: AcLDL = acetylated low-density lipoprotein; FACS = fluorescence-activated cell sorting; FEF = forced expiratory flow; FITC = fluorescein isothiocyanate; ILO = International Labor Organization; LD = linkage disequilibrium; MAF = minor allele frequency; MDM = monocyte-derived macrophage; MFI = median fluorescence intensity; MSR = macrophage scavenger receptor; *MSR1* = macrophage scavenger receptor-1 gene; NETT = National Emphysema Treatment Trial; PBS = phosphate-buffered saline; pp = percent predicted; rpm = revolutions per minute; RT = room temperature; SNP = single nucleotide polymorphism

COPD occurs in approximately 25% of smokers¹ in a pattern that is consistent with familial aggregation and genetic susceptibility.² Macrophages have an important role in the regulation of pulmonary inflammation and parenchymal destruction associated with COPD.³ Macrophages secrete elastases, tumor necrosis factor, and growth factors that may be important in the pathogenesis of COPD. In addition, macrophages

cluster in areas of tissue destruction.³⁻⁵ The presence of macrophages in the airways in COPD is inversely proportional to the level of FEV₁.⁶

Macrophage scavenger receptor-1 gene (*MSR1*) is in a genomic region that has been suggested to be linked to COPD susceptibility.⁷ This gene may be a potential candidate gene for COPD.⁸ *MSR1* is located on chromosome 8p22 and consists of 11 exons.⁹ Three

types of messenger RNAs are generated by alternate splicing from exon 9 that result in the two receptor proteins, macrophage scavenger receptor (MSR)1 and MSR2. Ten sequence variants in the gene, including one nonsense change (ex6R293X_C/T) and seven missense changes (ex3P36A_C/G, ex3S41Y_C/A, ex4V113A_T/C, ex4P174Y_G/T, ex6P275A_C/G, ex10G369S_G/A, and ex11H441R_A/G) have been reported.¹⁰ Polymorphism-176511_A/G is located in the promoter region of the gene and is thought to regulate production rate; IVS5-59_C/A is located in the intron and is thought to regulate splicing of the gene to regulate relative production of MSR proteins type 1 and type 2; ex6P275A_C/G (P275A) codes for the ligand binding site of MSR1. To evaluate the role of polymorphisms in the *MSR1* in COPD susceptibility and related measures of lung function, we genotyped a large, well-characterized population of at-risk smokers.¹¹ Functional assays were done on macrophages from donors with specific genotypes to determine the effect of *MSR1* single nucleotide polymorphisms (SNPs) on macrophages by examining *in vitro* survival, adhesion, and receptor expression.

MATERIALS AND METHODS

Study Population

Subjects were recruited from a cohort of tradesmen referred for a work-related, independent, medical evaluation.¹² Referrals were drawn from trade unions, television, and newspaper advertisements. Participants gave informed consent for their involvement, and the research protocol was reviewed and approved by the institutional review boards at Wake Forest University and Saint Louis University. As part of the referral process, an extensive questionnaire, a chest radiograph, and pulmonary function

tests were obtained. The questionnaire gathered information about prior employment, smoking history, and personal and family health histories. The questionnaire was self-administered prior to evaluation, and the physician examiner reviewed it at the time of examination. Chest radiographs were obtained and interpreted by a certified B-reader. Chest radiograph abnormalities were quantified according to the International Labor Organization (ILO) scoring system.¹³ Pulmonary function testing was performed according to the American Thoracic Society published guidelines.^{14,15} The FEV₁ used for analysis was the best of at least three replicates (within 5% of each other) FVC maneuvers. Subjects were asked to quantify their cigarette smoking as packs per day and ages of initiation and cessation of tobacco use. Subjects over age 40 with ≥ 20 pack-years of smoking were genotyped in this study. The presence of moderate or severe occupational-exposure-induced lung disease (ILO scores $> 1/1$), mesothelioma, an anticipated survival of < 1 year because of active cancer, or other chronic diseases were exclusion criteria. Because of the apparent overlapping susceptibility among smokers to both COPD and lung cancer, a medical history of lung cancer was not an exclusion criteria.

COPD Phenotype

The COPD phenotype, a composite variable, was based on the Global Initiative for Chronic Obstructive Lung Disease guidelines¹⁶ and defined as a FEV₁/FVC ratio $< 70\%$ and percent predicted (pp) FEV₁ $< 75\%$ in long-term smokers over the age of 40 years. Implicit in this definition is that subjects with COPD experience a decline in FEV₁ that is in excess of the age-related loss experienced by the general population over 30 years of age. This definition of COPD was more strict than the Global Initiative for Chronic Obstructive Lung Disease guideline criteria for stage 2 and above (FEV₁/FVC ratio $< 70\%$ and ppFEV₁ $< 80\%$) and was chosen based on the observation of Scanlon and coworkers¹⁷ that there is an inflection point on the rate of decline in the FEV₁ vs baseline ppFEV₁ curve in obstructed smokers whose FEV₁ is $< 75\%$ predicted. These data suggest that only obstructed smokers with a ppFEV₁ $< 75\%$ are at risk for the precipitous decline in FEV₁ that is characteristic of COPD. Controls consisted of long-term cigarette smokers with normal pulmonary function (FEV₁/FVC ratio $\geq 70\%$ and ppFEV₁ $\geq 80\%$). Smokers with an FEV₁/FVC ratio $\geq 70\%$ and ppFEV₁ $< 80\%$, or an FEV₁/FVC ratio $< 70\%$ and ppFEV₁ $\geq 75\%$ were excluded from categorical analyses (COPD vs unaffected smokers) because they could not be classified in terms of the presence or absence of COPD but were included in additional analyses of continuous variables (quantitative traits: ppFEV₁, ppFVC, FEV₁/FVC ratio, and pp forced expiratory flow [FEF]₂₅₋₇₅).

Genotyping Method

Samples were genotyped for -176511_A/G, ex4P174Y_G/T, IVS5-59_C/A, ex6P275A_C/G (rs3747531), ex6R293X_C/T (rs41341748), ex4V113A_T/C, and ex11H441R_A/G using the MassARRAY genotyping system (Sequenom; San Diego, CA), which uses a primer extension assay followed by mass spectrometry for oligonucleotide size determination. These genes were chosen because they were coding, promoter region, or intronic SNPs that could plausibly affect macrophage function. Genotypes were scored automatically using the SpectroTyper software (Sequenom) and checked with quality control samples (ie, duplicate DNA samples, negative controls) manually.

In Vitro Macrophage Functional Assays

Subject Identification and Selection: A separate group of volunteers were genotyped to identify a population that could be used

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to test the effects of the P275A SNP on function. Eight subjects out of 85 healthy, nonsmoking volunteers in their 20s and 30s were identified at the University of Montana as having the P275A_CG/GG mutation, with two subjects being homozygous (GG) and six subjects being heterozygous (CG). Eight wild-type (P275A_CC) subjects were selected at random to serve as a control group for the study. The University of Montana group was blinded for the genotypes of the subjects. The 16 selected subjects were recalled for an additional blood draw. One of the P275A_CG/GG subjects was unavailable for the second blood draw.

Monocyte-Derived Macrophage Isolation and Culture: Peripheral blood monocytes were isolated by density centrifugation and cultured with Ficoll-Histopaque followed by adherence to cell culture conditions. The blood sample was diluted 1:1 with sterile saline and placed in 50-mL polypropylene conical centrifuge tubes (VWR; West Chester, PA). Histopaque-1077 Hibri-Max (Sigma Chemical; St. Louis, MO) was layered below the blood at a volume of 13 mL by gravity release. The resulting gradient was centrifuged in a Forma model 5682 (ThermoForma; Marietta, OH) at 1,500 revolutions per minute (rpm) for 45 min at room temperature (RT). This centrifugation produced a layer of WBC (buffy coat) and blood platelets visible above the lower RBC pellet. This buffy-coat layer was carefully removed by 6-mL sterile plastic transfer pipettes (Samco; San Fernando, CA) and placed in 50-mL conical centrifuge tubes. Phosphate-buffered saline (PBS) with 1% bovine serum albumin was added up to 50 mL to dilute the sample. This was then centrifuged at 1,500 rpm for 5 min at RT. The resulting supernatant was aspirated and discarded while the cell pellet was resuspended in 1 mL RPMI tissue culture media (Mediatech; Herndon, VA) supplemented with 10% heat-inactivated human A/B sera (Invitrogen; Carlsbad, CA) and antibiotic/antimycotic cocktail (Mediatech). This cell suspension was then layered above 20 mL of heat-inactivated fetal bovine serum (Hyclone; Logan, UT) by slowly pipetting the cell suspension down the side of the 50-mL conical tube. This preparation was then centrifuged at 1,200 rpm for 10 min at RT to remove the blood platelets. The resulting supernatant was aspirated and discarded while the cell pellet (lymphocytes and monocytes) was resuspended in 2 mL RPMI tissue culture media as described above. A 40- μ L sample was removed and counted on a Z1 particle counter (Beckman/Coulter; Miami, FL). The 2-mL cell suspension was brought up to 10 mL in RPMI media and placed in a 75-cm² tissue culture flask (Corning; Corning, NY), and incubated for 1 h at 37°C in a water-jacketed CO₂ incubator (ThermoForma). After 1 h, the nonadherent cell fraction (lymphocytes) was removed, centrifuged, and counted as stated above. The adherent cell fraction (monocytes) was then cultured in 20 mL RPMI media described above for 10 additional days.

Monocyte-Derived Macrophages: Five days into the 10-day monocyte-derived macrophages (MDM) culture, the nonadherent cells and debris were removed by aspiration and discarded. The adherent cell fraction was trypsinized (EDTA/trypsin solution; Mediatech) for 10 min at 37°C to dislodge all adherent cells. The trypsin was then inactivated by culture media. Any remaining adherent cells were scraped with a rubber cell scraper (Corning). The resulting cell suspension was centrifuged at 1,500 rpm for 5 min at RT. The supernatant was aspirated and discarded while the cell pellet was resuspended in 1 mL RPMI tissue culture media and counted as described above. The 1-mL cell suspension was brought up to 5 mL in RPMI media, placed in a 25-cm² tissue culture flask (Corning), and incubated for an additional 5 days. The adherent cell removal was done to make the 10-day cell retrieval less difficult and harmful to the MDM. The final cell retrieval (day 10) was identical to the day 5 process with trypsinization, scraping, and

counting of the MDM, with the exception of allowing the cells to readhere. The MDM were suspended in tissue culture media at 10⁶ cells/mL and stained for MSR1 surface expression.

MDM Survival: Total cell number in culture is related to the balance of cell proliferation and apoptosis. Macrophages are terminally differentiated cells.¹⁸ Because these cells do not divide^{19,20} except in rare instances when growth factors are provided to stimulate them, total cell number in culture is related directly to survival.

MDM Adherence Scale: The effectiveness of the trypsin in dislodging the MDM was determined by quantifying the ratio of adherent cells to loose cells, where 1 = more than 75% removed; 2 = between 50% and 75% removed; 3 = between 25% and 50% removed; and 4 = less than 25% of cells removed by trypsin. This was accomplished by counting loose vs adherent cells in a random field using a hand counter stopping on 100 counts. This assessment was developed exclusively for this study when it was apparent from the original two samples processed that the MDM adherence was highly variable from subject to subject. It is referred to as a subjective scale because only one rater was used throughout the study and the interrater reliability was not established. It is important to note that the lone rater was blind to the experimental conditions.

MSR1 Cell Surface Expression: Approximately 3×10^5 MDM cells (which varied depending on cell yield) were incubated with unconjugated primary antibodies for MSR1 (mouse monoclonal, 1:100 dilution; R & D Systems; Minneapolis, MN). Cells were then washed with PBS and resuspended in culture media. The MDM were incubated with conjugated secondary antibodies against mouse IgG (fluorescein isothiocyanate [FITC], 1:1,000 dilution; Molecular Probes, Invitrogen; Carlsbad, CA) for 30 min at RT. Cells were washed in PBS and pipetted into filter-top tubes (BD; Franklin Lakes, NJ) for fluorescence-activated cell sorting (FACS) analysis on a FACS Caliber (Beckman/Coulter). Controls included unstained cells and secondary antibody only. Data were expressed as percentage of MDM expressing MSR1, and relative intensity of expression/cell (median fluorescence intensity [MFI]).

Fluorescent Acetylated Low-Density Lipoprotein Uptake: Approximately 3×10^5 MDM cells (which varied depending on cell yield) were incubated with FITC-conjugated acetylated low-density lipoprotein (AcLDL) (1:2,000 dilution; Molecular Probes, Invitrogen) for 1 h in an end-over-end suspension culture on a labquake shaker (Labindustries; Berkeley, CA) at 37°C. Following the incubation period, the cells were washed in PBS and transferred to filter-top tubes for FACS analysis. Data were expressed as MFI on the FITC channel. Controls included cells without AcLDL stimulation.

Statistical Methods

Genetic Studies: For quality control, all polymorphisms were tested for consistency with Hardy-Weinberg equilibrium using χ^2 tests. The frequency of MSR1 genotypes in subjects with COPD and unaffected control smokers was compared using a χ^2 test. For the COPD phenotype and pulmonary function measures (ppFEV₁, ppFVC, FEV₁/FVC ratio, and ppFEF₂₅₋₇₅), associations for the genotypes of SNPs were assessed using logistic and linear regressions, respectively. These models were adjusted for sex, age, and pack-year smoked, and statistical analyses were performed using SAS software (SAS Institute; Cary, NC).

In Vitro Studies: All data were analyzed by independent-samples *t* tests, with the exception of the subjective scale data that was collected on how strongly the MDM cells were adhering to the tissue

culture plates. In this case, the nonparametric Mann-Whitney test was used instead. All analyses were two tailed, with the exception of the binding/uptake studies (eg, silica, AcLDL, *E coli*), which were one tailed because of the directional hypothesis that binding would be greater in the mutant MSR1 cells. Statistical significance was determined to be type 1 error at $<5\%$ ($P < .05$). MFI was preferred to mean fluorescence intensity because the distribution characteristics (log normal) indicated that the median was a better measure of central tendency. Statistical outliers were determined by the Grubb test. Only one value was determined to be an extreme deviate value and it was excluded from the analysis. Data analyses and graphics were performed on PRISM software (version 4.0b; GraphPad; San Diego, CA).

RESULTS

Population Studies

DNA was available from approximately 3,000 of the $>5,000$ subjects enrolled in this cohort. The cohort was $>90\%$ white males, and 68% smoked.¹² The enrollment rate was $>95\%$ of individuals evaluated for occupational exposure. Because of the white majority of the population, genetic analysis was limited to this race. The clinical characteristics of the groups with COPD and unaffected smokers are shown in Table 1. Of the 714 subjects who met the inclusion criteria and were genotyped, 275 met COPD case definition criteria, 212 were unaffected smoking controls, and 227 fell into the group excluded from categoric analyses ($FEV_1/FVC \geq 70\%$ and $ppFEV_1 < 80\%$ or $FEV_1/FVC < 70\%$ and $ppFEV_1 \geq 75\%$). Five of the seven SNPs had a minor allele frequency (MAF) <0.05 and showed no association with the COPD phenotype (Fig 1). These were not further analyzed. Genotype frequencies for the two more common SNPs, -176511_A/G (MAF = 0.08) and P275A (MAF = 0.06), were consistent with Hardy-Weinberg equilibrium. We observed significant evidence for an association between SNP P275A ($P < .005$) and susceptibility to

COPD when comparing subjects with the COPD phenotype with the smoking unaffected controls (Table 2). For P275A, heterozygote subjects (CG) were more frequent in the COPD group (13.1%) compared with unaffected smokers (5.2%). No homozygotes were seen in this population. Inclusion of potential confounders (age, sex, pack-years smoked, smoking status [current vs ex-smoker], and ILO score) did not affect the results. The adjusted (for pack-years smoked, sex, and age) odds ratio (95% CI) for the CG genotype vs GG genotype was 2.32 (1.24, 4.33). For the quantitative lung measures analyzed in the total population of smokers, subjects with the CG genotype at P275A had a lower $ppFEV_1$, FEV_1/FVC ratio, and $ppFEF_{25-75}$ compared with the CC subjects. Although the difference was only significant for FEF_{25-75} ($P = .03$), a consistent trend was observed (Table 3). None of the subjects identified as COPD or unaffected smoking controls had a GG genotype. However, one normal volunteer donor used in the *in vitro* studies did have the GG genotype.

In Vitro Studies

MDM Cell Number: Despite isolation and inoculation of equal numbers of monocytes from both groups into the culture media, there were nearly twice as many MDM in culture from MSR1 P275A_CG/GG donors as from P275A_CC donors throughout the 10-day culture period (Fig 2). The mean count was 3.25×10^6 cells for P275A_CG/GG donors and 1.93×10^6 cells for CC donors ($P < .05$). The disparity in total cell number was apparent by day 5, with the CG/GG mean count at 4.94×10^6 cells and the CC mean count at 2.95×10^6 cells ($P < .05$). The total number of lymphocytes and monocytes on the first day were statistically identical between the two study groups (Fig 2 *inset*).

Table 1—Characteristics of Subjects With COPD and Smokers With Normal Pulmonary Function

Characteristic	Unaffected Smoking Controls ($ppFEV_1 \geq 80$ and FEV_1/FVC (%) ≥ 70) (n = 212)	COPD ($ppFEV_1 < 75$ and FEV_1/FVC (%) < 70) (n = 275)	Unclassified (n = 227)	P Value ^a
Age	63.1 (8.7)	67.4 (8.5)	65.4 (9.1)	$<.001$
% male	95.7	97.4	97.4	.50
Pack-years	44.6 (25.7)	58.1 (31.4)	51.8 (27.2)	$<.001$
FEV_1/FVC (%)	78.8 (6.5)	54.6 (11.0)	71.8 (7.9)	$<.001$
$ppFEV_1$	94.9 (9.7)	50.7 (14.4)	74.1 (15.0)	$<.001$
$ppFVC$	94.4 (11.0)	70.9 (15.1)	81.8 (20.6)	$<.001$
$ppFEF_{25-75}$	81.8 (38.8)	20.6 (24.2)	51.9 (17.3)	$<.001$

Data are presented as mean (SD) unless otherwise indicated. Of 714 whites who smoked ≥ 20 pack-years and were older than 40 y, 227 fell into the group excluded from categoric analyses ($FEV_1/FVC \geq 70\%$ and $ppFEV_1 < 80\%$ or $FEV_1/FVC < 70\%$ and $ppFEV_1 \geq 75\%$). The analysis shows significant differences in age and pack-years smoked. $ppFEV_1$, FEV_1/FVC ratio, and $ppFEF_{25-75}$ were different because of selection criteria. pp = percent predicted; FEF = forced expiratory flow.

^aWilcoxon two-sample test.

FIGURE 1. Five of the seven single nucleotide polymorphisms (SNPs) had a minor allele frequency <0.05 and were not analyzed further. A significant association between SNP P275A ($*P < .005$) and susceptibility to COPD is demonstrated.

MSR1 Expression on MDM: MSR1 expression was significantly greater in MDM cells from P275A_CG/GG donors compared with the wild-type P275A_CC donors (Fig 3A). MSR1 expression was noted on 88.6% of MDM cells from CG/GG donors and on only 74.6% of MDM cells from CC donors ($P < .05$). The number of MSR1 per cell ($P < .05$), as well as the percentage of cells expressing MSR1, increased (Fig 3B) when MSR1 receptor expression was expressed as a function of MFI.

MDM Cell Adherence: MDM from MSR1 P275A_CG/GG donors were more adherent to the plastic tissue culture surface on day 10 than were the MDM from P275A_CC donors (Fig 4). The median score for MDM from the P275A_CG/GG subjects was three, whereas the median score for MDM from the control subjects was one ($P < .05$).

AcLDL Binding: There was a nonsignificant ($P = .08$) increase in AcLDL uptake by MDM from MSR1 P275A_CG/GG donors compared with MDM from P275A_CC donors (Fig 5). There was a doubling of

the amount of AcLDL taken up by MDM from P275A_CG/GG subjects (average MFI = 2,206) compared with MDM from CC control subjects (average MFI = 1,139).

DISCUSSION

In this study, we evaluated the role of MSR1 variation in COPD susceptibility and related measures of lung function, and determined the effect of MSR1 genotype on *in vitro* macrophage function from donors with at-risk genotypes. We found that a MSR1-coding SNP, P275A, was associated with a COPD phenotype and measures of lung function (trend for FEV₁ and FEV₁/FVC and significance for ppFEF₂₅₋₇₅). The P275A_CG/GG genotype was also associated with increases in maintenance of cell number in culture (increased survival/reduced apoptosis), MSR1 receptor expression, and adhesion of macrophages to plastic *in vitro*. There was also a nonsignificant (probably due to a lack of statistical power) increase in uptake of AcLDL by macrophages from P275A_CG/GG donors. Evidence for an association between the MSR1-coding SNP, P275A, with COPD was demonstrated in three different and complimentary ways. P275A was associated with the COPD phenotype, with *in vitro* macrophage functional abnormalities, and with related measures of lung function. One previous study examined the association of polymorphisms in MSR1 with susceptibility to COPD but did not evaluate the functional sequelae of these polymorphisms.⁸ Based on previous results from their family study in the Boston Early Onset COPD program, Hersh and coworkers⁸ fine-mapped the linkage region on chromosome 8p and evaluated two positional candidate genes. No significant results were observed for DEFB1, but for MSR1, the same polymorphism significant in our study, P275A, was significantly associated with two measures of airflow obstruction ($P \leq .02$), although they found an opposite effect because carriers of the rare allele had a lower risk of airflow obstruction. To replicate these results, the authors genotyped participants from the National Emphysema Treatment Trial (NETT) and the Boston Normative Aging Study. Using a case-control format, they found weak evidence for an association ($P = .04$), with the minor allele being underrepresented in the cases, and concluded that there was marginal evidence for an association between MSR1 and susceptibility to COPD.

Several design-related differences exist between our study and that of Hersh and coworkers,⁸ which may explain our divergent findings. Both studies have similar sample sizes but differed in disease severity and choice of controls. COPD was of earlier onset

Table 2—Associations Between SNPs in MSR1 and COPD

SNP	Genotype	COPD (n = 275)		Controls (n = 212)		P Value ^a
		No.	%	No.	%	
P275A ^a	CG	36	13.1	11	5.2	$<.005$
	CC	239	86.9	201	94.8	...
-176511 ^{ab}	AG, GG	47	17.1	34	16.0	.74
	AA	228	82.9	178	84.0	...

SNP = single nucleotide polymorphism; MSR1 = macrophage scavenger receptor-1 gene.

^aThese SNPs had a minor allele frequency >0.05 .

^bThree individuals in the COPD group and one individual in the control group had the GG genotype. Because of this low frequency, GG genotypes were combined with the heterozygous genotypes.

Table 3—Estimated Mean Pulmonary Function Measures for Genotypes of SNP P275A in MSR1 Gene

Genotype	ppFEV ₁	P Value ^a	ppFVC	P Value ^a	Ratio	P Value ^a	ppFEF ₂₅₋₇₅	P Value ^a
CG (n = 81, 11.3%)	68.0	.15	79.6	.29	66.2	.46	45.7	.03
CC (n = 633, 88.7%)	71.7		81.9		67.4		53.4	

Ratio = FEV₁/FVC ratio. See Tables 1 and 2 for expansion of other abbreviations.

^aGeneralized linear models, adjusted for sex, age, and pack-year smoked.

and tended to be more severe in both the NETT and Boston Early Onset Cohort than in our population.²¹ The controls had ≥ 10 pack-years of smoking (not 20, as in our study). Thus, the phenotype of the affected subjects and controls enrolled in their studies may have been different from ours. We studied a large, well-characterized population of at-risk smokers derived from one region in the United States, making it more likely that, as a result of migration patterns, the subjects and controls would come from genetically similar backgrounds. The NETT population (n = 304) used by Hersh et al⁸ recruited subjects from multiple centers throughout the United States to study the safety and efficacy of lung reduction surgery in severe COPD,²² making it a more genetically heterogeneous population. The controls (n = 441) for the Hersh et al⁸ study were derived from a separate cohort from the NETT altogether. Controls for the NETT population in the Hersh⁸ study were taken from the Normative Aging Study cohort which was a population derived primarily from the Boston area designed to evaluate age-related changes in lung function. Therefore, the Hersh⁸ study used affected subjects from across the United States and compared them with control sub-

jects derived from the Boston area, which could have led to comparison of two genetically heterogeneous populations. Finally, the divergent findings between those of Hersh and coworkers⁸ and our data may be that the SNP responsible for the association is near to, or in linkage disequilibrium (LD), with the P275A SNP.

Current evidence indicates that macrophages play an important role in the regulation of the inflammation and tissue destruction associated with COPD.³ The number of macrophages increases in lung parenchyma and^{4,5} small airways,^{4,6} and within the BAL fluid of patients with COPD.²³ Furthermore, macrophages have been found in sites of lung remodeling or destruction^{5,24} and their number has been shown to correlate with the severity of COPD.^{6,25} These findings suggest that mechanisms leading to accumulation of macrophages in the lung could contribute to COPD. In recent reviews, both Tetley²⁶ and Barnes²⁷ speculated that macrophage accumulation could result from three different mechanisms: prolonged survival, increased recruitment, or impaired clearance. All three of these mechanisms may occur in the lungs of smokers with COPD. The normal clearance of dead and dying macrophages through the mucociliary

FIGURE 2. Cell counts on day 5 and day 10 of the study. Mean $\pm$ SEM number of adherent cells at day 5 and at day 10. Asterisk (*) indicates statistical significance at $P < .05$ compared with corresponding P275A_C controls. Inset: Mean $\pm$ SEM total number of cells isolated on first day with lymphocyte and monocyte fractions indicated. Sample size was n = 8 for the P275A_CC group and n = 7 for the P275A_CG/GG group. Lymph = lymphocyte; Mono = monocyte.

FIGURE 3. MSR1 expression at day 10. Mean $\pm$ SEM percent cells expressing MSR1 surface marker (A). The relative intensity of MSR1 expression, expressed as MFI, is likewise increased in the P275A_CG/GG group (B). Asterisk (*) indicates statistical significance at $P < .05$ compared with P275A_CC controls. Sample size was $n = 8$ for the P275A_CC group and $n = 7$ for the P275A_CG/GG group. MFI = median fluorescence intensity; MSR1 = macrophage scavenger receptor 1.

system is impaired in smokers with COPD. Apoptosis is reduced in alveolar macrophages of smokers, which may lead to prolonged survival.²⁸ There is also evidence for increased recruitment of monocytes and enhanced retention of macrophages in the lungs of subjects with COPD. Recruitment of peripheral blood monocytes by chemotaxis is increased in smokers with COPD compared with “healthy smokers” and controls.²⁹ Kirkham and coworkers³⁰ have shown that enhanced retention of macrophages in smokers with COPD may be related to MSR1. They have shown that macrophages adhere to cigarette-smoke-damaged type 4 collagen through MSR1 and then are resistant to further chemotactic stimuli. In addition to its role in adherence to cigarette-smoke-damaged type 4 collagen, MSR1 has a major role in adhesion of macrophages to extracellular matrix.³¹ Its ligands include denatured type 4 collagen,³² cigarette-smoke-modified type 4 collagen,³⁰ cells undergoing apoptosis,³² activated lymphocytes, and both gram-negative and gram-positive bacteria.^{33–35} Furthermore, in a recent study, Heguy and colleagues³⁶ showed that *MSR1* is upregulated in smokers.

The P275A_CG/GG genotype in our study was associated with an increase in the MSR1 receptor number and increased MDM adhesion to plastic *in vitro*. These two findings provide a mechanism for not only increased recruitment, but also decreased clearance of macrophages in the lung in COPD. The alterations in adhesion seen in P275A donors were likely due to this gene variation, either directly affecting adherence or through the increase in receptor number. Although the scoring for adherence was subjective, the scorer was blinded to the genotype conditions, minimizing any experimental bias.

This study shows that there were more MDM from P275A_CG/GG donors than controls in culture at all time points evaluated after inoculation of equal num-

bers of cells from both groups. This increased macrophage cell count was not due to differences in the number of cells initially isolated. Because these cells are known not to proliferate,^{18,19,20} this increased number of cells seen in culture from P275A_CG/GG donors must be the result of increased survival or reduced apoptosis. Although it is possible that the increased adhesion to plastic may account for the observed prolonged survival *in vitro* of MDM from P275A_CG/GG donors, the more likely cause accounting for the approximately twofold increase in the

FIGURE 4. Relative strength of MDM cell adherence on day 10; 1 = $> 75\%$ of cells removed by trypsin; 2 = 50% to 75% of cells removed by trypsin; 3 = 25% to 50% of cells removed by trypsin; 4 = $< 25\%$ of cells removed by trypsin. In all cases, remainder was removed by scraping. Horizontal line indicates median. Asterisk (*) indicates statistical significance at $P < .05$ compared with *MSR1* gene P275A_CC controls by Mann-Whitney test. Sample size was $n = 8$ for the P275A_CC group and $n = 7$ for the *MSR1* P275A_CG/GG group. MDM = monocyte-derived macrophage. See Figure 3 legend for expansion of other abbreviation.

FIGURE 5. Uptake of fluorescein isothiocyanate-labeled AcLDL after 1-h suspension culture. Mean $\pm$ SEM MFI. ($P = .08$ one-tailed t test.) Sample size was $n = 8$ for the *MSR1* P275A_CC group and $n = 7$ for the P275A_CG/GG group. AcLDL = acetylated low-density lipoprotein. See Figure 3 legend for expansion of other abbreviation.

number of macrophages surviving was the presence of the P275A mutation. This polymorphism (SNP) or another gene variation in LD with this SNP is a potential mechanism for this finding. There are three other SNPs (rs970440, 351540, and 351575) in strong LD ($r^2 \geq 0.8$) with P275A (rs3747531). These three SNPs are within the intronic area of the gene and are therefore potentially less important than P275A, which resides in the area of the gene that is associated with an amino acid change in the receptor binding site.⁹ Its strategic location and the cumulative findings of increases in maintenance of cell number in culture (increased survival/reduced apoptosis), *MSR1* receptor expression, number of receptors/cells, and adhesion to plastic *in vitro*, regardless of their potential for interaction, is a reasonable accounting mechanism for the association of this SNP with COPD.

A possible limitation of this research is the study of macrophages matured from peripheral blood monocytes. Although lung macrophages may have been a more appropriate model, it would have required conducting invasive BAL and subject willingness. Therefore, MDM, which are an accepted model of macrophages, were used in this screening study. Another possible limitation is the potential for exposure to mixed dust in the population studied. The prevalence of cigarette smoking in the United States is currently 23%.³⁷ However, the prevalence of cigarette smokers among blue collar workers is incrementally greater than among the general public, with prevalence in some trades such as roofers and stone masons near or in excess of 60%.³⁸ Therefore, any population of smokers used in epidemiologic research is likely to be highly enriched in

blue collar workers with mixed dust exposure. Referrals for occupational exposure that comprised our cohort were blue collar workers involved in the building trades, in construction, or in foundries and refineries,¹² similar to those enrolled in other COPD cohort studies. In contrast to subjects currently enrolled in these other COPD studies, each subject in our cohort had a chest radiograph that was read by a certified B-reader, so we were able to confidently exclude subjects with moderate and severe exposure-related lung abnormalities.

Usually, one expects the degree of association to be lower for qualitative traits than for quantitative traits. The fact that the significance level for the association between P275A and the qualitative phenotype (COPD) is higher compared with the quantitative traits (lung function measures) is an important finding in this study. One explanation for this might be that COPD is a different and distinguished entity compared with other smoke-related lung injury (eg, subjects with restriction) because COPD was defined by two different measures of lung function (ppFEV₁ and FEV₁/FVC). This definition excluded subjects who were not classified as COPD or unaffected smokers. In fact, one of the strategies in choosing a phenotype for susceptibility genetic studies is to use the two ends of a phenotype spectrum. This will help eliminate the gray zone (unclassified group in our study) in the phenotype. Therefore, the higher level of significance for COPD compared with lung function measures, which included all smoke-related, lung-injured subjects, is more evidence that *MSR1* is an important gene in COPD. Because this is a hypothesis-driven genetic association study, correction for multiple comparisons is controversial. The two SNPs we analyzed in *MSR1* had a D-prime of 0.45 and r-square of zero. Using a conservative Bonferroni adjustment for multiple testing would result in a P value still $< .01$ for P275A. Even though *MSR1* was chosen because of its chromosomal location and report of association in another study, a related issue is the possible effect of multiple testing because of other genes tested in this population. Our current result of < 0.005 (or < 0.01) would remain significant ($P < .05$) with correction for at least 10 tests, which we feel is appropriate, given the usual LD structure in SNPs tested in genes that we have reported.^{11,39} However, the accumulation of the effects of multiple testing as additional candidate genes are tested in the same population remains an issue, and replication of these results will be important. We did not test for population stratification; however, given that our *in vitro* functional assays are consistent with our *in vivo* data, it is unlikely that the findings were the result of population stratification.

COPD is a disease that affects 20 to 30 million Americans.¹⁶ Because of the recent rapid increase in the incidence of COPD, it is anticipated that this disease will be an increasing public health problem worldwide during the next decade.¹⁶ Although there is a significant interaction between exposure to tobacco smoke (duration and amount of cigarettes smoked) and FEV₁, the actual number of cigarettes smoked is not predictive of developing COPD, thus implicating other genetic or environmental causes. The minor allele of *MSR1* P275A SNP was present in 13% of smokers with COPD in our cohort.

CONCLUSIONS

One in four smokers develops COPD, often in a familial pattern. Macrophages play an important role in the regulation of the pulmonary inflammation and parenchymal destruction associated with COPD. A coding SNP (P275A) in the binding site of a macrophage cell surface receptor that was associated with susceptibility to COPD in smokers ($P < .005$) decreases in ppFEV₁, FEV₁/FVC, and ppFEF₂₅₋₇₅ ($P = .03$) and increases in maintenance of cell number in culture (increased survival/reduced apoptosis), *MSR1* expression, and adhesion of macrophages to plastic *in vitro* ($P < .05$). The *MSR1* association with COPD susceptibility, COPD-related measures of lung function, and abnormalities of macrophage function may account for significant COPD morbidity.

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Mr Hamilton Jr: contributed to choosing and performing the *in vitro* macrophage assays.

Dr Zheng: contributed to developing and performing the genotyping assays.

Dr Sadeghnejad: contributed to developing and performing the statistical methods and analysis of the genetic data.

Dr Sterling: provided support in the development of the population.

Dr Xu: contributed to overseeing the genotyping assays.

Dr Meyers: contributed to overseeing the statistical methods and analysis of the genetic data.

Dr Bleecker: contributed to interpreting clinical and genetic data.

Dr Holian: contributed to choosing and performing the *in vitro* macrophage assays.

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