A functional promoter polymorphism in *monocyte chemoattractant protein–1* is associated with increased susceptibility to pulmonary tuberculosis

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We examined the distribution of single nucleotide polymorphisms (SNPs) in *nitric oxide synthase 2A*, *monocyte chemoattractant protein–1* (*MCP-1*), *regulated on activation, normal T cell expressed and secreted*, and *macrophage inflammatory protein–1α* genes in tuberculosis patients and healthy controls from Mexico. The odds of developing tuberculosis were 2.3- and 5.4-fold higher in carriers of *MCP-1* genotypes *AG* and *GG* than in homozygous *AA*. Cases of homozygous *GG* had the highest plasma levels of MCP-1 and the lowest plasma levels of IL-12p40, and these values were negatively correlated. Furthermore, stimulation of monocytes from healthy carriers of the genotype *GG* with *Mycobacterium tuberculosis* antigens yielded higher MCP-1 and lower IL-12p40 concentrations than parallel experiments with monocytes from homozygous *AA*. Addition of anti–MCP-1 increased IL-12p40 levels in cultures of *M. tuberculosis*–stimulated monocytes from homozygous *GG*, and addition of exogenous MCP-1 reduced IL-12p40 production by *M. tuberculosis*–stimulated monocytes from homozygous *AA*. Furthermore, we could replicate our results in Korean subjects, in whom the odds of developing tuberculosis were 2.8- and 6.9-fold higher in carriers of *MCP-1* genotypes *AG* and *GG* than in homozygous *AA*. Our findings suggest that persons bearing the *MCP-1* genotype *GG* produce high concentrations of MCP-1, which inhibits production of IL-12p40 in response to *M. tuberculosis* and increases the likelihood that *M. tuberculosis* infection will progress to active pulmonary tuberculosis.

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Abbreviations used: ANOVA, analysis of variance; BCG, Bacillus Calmette-Guerin; BMI, body mass index; CI, confidence interval; *MCP-1*, *monocyte chemoattractant protein–1*; *MIP-1α*, *macrophage inflammatory protein–1α*; *NOS2A*, *nitric oxide synthase 2A*; OR, odds ratio; *RANTES*, *regulated on activation, normal T cell expressed and secreted*; SNP, single nucleotide polymorphism.

Studies of monozygotic and dizygotic twins have demonstrated that genetic factors contribute considerably to the development of tuberculosis (1, 2). The 17q11.2 chromosomal region has been linked to susceptibility to tuberculosis (3, 4) and includes genes encoding for several chemokines that may contribute to immunity against tuberculosis. One gene encodes *monocyte chemoattractant protein–1* (*MCP-1*), a chemoattractant for monocytes and T lymphocytes, which are central components of the granulomatous response (5). Other β chemokine genes in this region are *macrophage inflammatory protein–1α* (*MIP-1α*), and *regulated on activation, normal T cell expressed and secreted* (*RANTES*), which are involved in recruitment of T cells to inflammatory sites (6), activation of T cells (7), and inhibition of intracellular growth of *Mycobacterium tuberculosis* (8). Another interesting gene in this region is the *nitric oxide synthase 2A* (*NOS2A*) gene, which generates nitric oxide, a molecule with antimicrobial activity against *M. tuberculosis* (9).

Single nucleotide polymorphisms (SNPs) in the *MCP-1* and *NOS2A* promoters influence expression of these genes (10, 11), and

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SNPs in the *RANTES* and *MIP-1α* genes are associated with susceptibility to HIV infection (12). We therefore performed a study to determine whether susceptibility to development of tuberculosis after infection was associated with any of these four SNPs and to delineate the mechanisms underlying this susceptibility.

RESULTS

Features of clinical and genomic controls

We recruited 445 new sputum smear–positive cases with culture-confirmed tuberculosis and 518 healthy controls in Mexico. All cases and controls were recruited through the World Health Organization's Mexican DOTS program for early detection of new tuberculosis cases (13), in which ~95% of cases are newly diagnosed and only 5% are cases of tuberculosis relapses (13).

Genotyping was successful in 98% of tuberculosis patients and healthy controls. Mexican controls were stratified into 334 healthy tuberculin reactors and 176 tuberculin-negative persons. The three groups were similar in demographics, body mass index ([BMI] before development of disease in the case of tuberculosis patients), household incomes, and consumption of cigarettes and alcohol (Table I). The absence of heavy cigarette or alcohol use was probably because of the exclusion of persons with diseases associated with these habits.

Chest radiographs showed alveolar infiltrates in all 435 tuberculosis patients, and hilar adenopathy was present in 417 (96%) cases from Mexico. No patients had a history of tuberculosis or previous treatment for tuberculosis. They were in contact with an individual with active tuberculosis for a period of no more than 8 mo before the symptoms were evident. None of them had other medical conditions affecting immunity. These clinical and epidemiological findings are consistent with the fact that our Mexican patients are new tuberculosis cases.

Mexican Mestizos from Mexico D.F. (the Federal District) have an admixture of Spanish (50.03 ± 4.11%), Amerindian (49.03 ± 3.76%), and African (0.94 ± 1.27%) traits (14). Thus, to determine whether our cases and controls were homogeneous in terms of admixture we genotyped 30 unlinked SNPs as genomic controls (15, 16). These SNP were not associated with disease and all of them were in Hardy-Weinberg equilibrium (Table S1, available at http://www.jem.org/cgi/content/full/jem.20050126/DC1).

Thus, our demographic and genomic control data indicate that it is unlikely that our results are caused by selection or information bias (including genotyping errors) (17), an unadjusted known confounder, or population stratification.

To confirm our findings in Mexicans in an ethnically distinct population, we studied subjects from Korea, including 129 with tuberculosis and 162 healthy controls. Demographic information of this sample is provided in Table S2 (available at http://www.jem.org/cgi/content/full/jem.20050126/DC1). There were no important differences in age, gender distribution, or BMI between cases and controls (Table S2).

Table I. Demographic and clinical features of tuberculosis cases and controls from Mexico[a]

Parameter	Tuberculosis (n = 435)	Healthy tuberculin positive (n = 334)	Healthy tuberculin negative (n = 176)
Gender			
Male, n (%)	296 (68)	230 (69)	122 (66)
Age (yr), mean ± SD			
Female	36 ± 8	34 ± 9	33 ± 11
Male	37 ± 5	38 ± 3	37 ± 6
BMI[b] (kg/m^2), mean ± SD			
Female	25.2 ± 4.5	26.3 ± 3.2	27.1 ± 3.1
Male	24.8 ± 5.1	25.5 ± 4.2	26.5 ± 2.6
Household income (dollars/yr), mean ± SD	2,633 ± 633	2,701 ± 461	2,714 ± 416
Smokers, n (%)[c]			
Female	11 (8)	7 (7)	5 (9)
Male	59 (20)	53 (23)	39 (22)
Alcohol use, n (%)[d]			
Female	7 (5)	6 (6)	2 (3)
Male	21 (7)	18 (8)	7(6)

[a]The distribution of categorical variables was compared by χ^2 or Fisher's exact tests, and that of continuous variables was done by one-way ANOVA. There were no significant differences between groups for any of the parameters.
[b]BMI was based on weight (self-reported before the development of disease in tuberculosis patients) and height (measured by a nurse).
[c]All smokers reported consumption of <6 cigarettes per day.
[d]All alcohol users reported consumption of <2 drinks per day.

The −2518 *MCP-1* promoter polymorphism is associated with susceptibility to pulmonary tuberculosis

In Mexican control subjects, genotypes at the four selected loci tested in the 17q11.2 chromosomal region were in Hardy-Weinberg equilibrium. None of the *NOS2A*, *RANTES*, and *MIP-1α* alleles (Table II) or genotypes (unpublished data) were associated with tuberculosis. In contrast, allele *G* of the *MCP-1* gene was strongly associated with tuberculosis compared with healthy tuberculin reactors, with a significant χ^2 of 12.9 ($P = 0.0003$), as corrected for population stratification/admixture (divided by $\lambda = 1.332$) and for the number of comparisons, and an odds ratio (OR) of 2.43 (95% confidence interval [CI] = 1.96–3.02). Similar results were obtained by comparing tuberculosis patients with healthy tuberculin-negative persons (corrected χ^2 = 9.08; $P = 0.0026$; OR = 2.45; 95% CI = 1.88–3.19; Tables II and III). Carriers of *MCP-1* genotypes *AG* and *GG* were significantly overrepresented among tuberculosis cases compared with healthy tuberculin reactors and tuberculin-negative controls (Table II). A trend test was also significant ($P < 0.00001$ for both comparisons), indicating that the *MCP-1*

Table II. The allele *G* of the *MCP-1* promoter region is associated with development of pulmonary tuberculosis (Mexican sample)[a]

Allele	TB[b] (870 alleles)	PPD+[c] (668 alleles)	TB versus PPD+	PPD−[d] (352 alleles)	TB versus PPD−
	n (frequency)	*n* (frequency)		*n* (frequency)	
NOS2A-954					
C	824 (0.95)	627 (0.94)	Not significant	329 (0.93)	Not significant
G	46 (0.05)	41 (0.06)		23 (0.07)	
MCP-1 −2518					
A	244 (0.28)	325 (0.49)		172 (0.49)	
G	626 (0.72)	343 (0.51)	$\chi^2 = 12.9$ P = 0.0003	180 (0.51)	$\chi^2 = 9.08$ P = 0.0026
RANTES-471					
A	234 (0.27)	179 (0.27)	Not significant	93 (0.26)	Not significant
G	636 (0.73)	489 (0.73)		259 (0.74)	
MIP-1α-459					
C	744 (0.86)	567 (0.85)	Not significant	298 (0.87)	Not significant
T	126 (0.14)	101 (0.15)		54 (0.13)	

[a]*MCP-1* alleles were typed by amplification fragment length polymorphism analysis. *NOS2A*, *RANTES*, and *MIP-1α* alleles were typed by amplification-created restriction site analysis, as detailed in Materials and methods. Groups were compared by χ^2 analysis. χ^2 values were corrected for population stratification and for the number of comparisons.
[b]TB = new cases of pulmonary tuberculosis.
[c]PPD+ = healthy tuberculin reactors.
[d]PPD− = healthy tuberculin-negative persons.

allele *G* has a dose effect. Indeed, the ORs for heterozygous *AG* in tuberculosis cases versus tuberculin-positive and -negative controls were 2.1 and 2.3, respectively, and increased to 5.4 and 5.5, respectively, for the comparison of homozygous *GG* (Table III).

Koreans have an admixture of two Asian populations of Mongolian origin, with 55% Northern Asian and 45% Southern Asian components (18). Thus, Koreans are much more ethnically homogeneous than Mexican Mestizos, and correction for population stratification/admixture was not applied to this sample. In Korean control subjects, genotypes of the *MCP-1* gene were in Hardy-Weinberg equilibrium. As in Mexicans, allele *G* of the *MCP-1* gene was strongly associated with tuberculosis compared with healthy controls, with a significant χ^2 of 32.28 (P = 0.0001) and an OR of 2.63 (95% CI = 1.85–3.73; Table IV). As in Mexicans, carriers of *MCP-1* genotypes *AG* and *GG* were significantly overrepresented among tuberculosis cases as compared with healthy controls (Table V). A significant dose effect of the *MCP-1* allele *G* was also observed in Koreans, because the OR for heterozygous *AG* in tuberculosis cases versus healthy controls was 2.8 and strongly increased to 6.9 for the comparison of homozygous *GG* (Table V).

Tuberculosis patients with the *MCP-1 GG* genotype have the highest MCP-1 and the lowest IL-12p40 plasma concentrations

MCP-1 plasma levels were significantly higher in 145 tuberculosis patients from Mexico than in 102 controls (80 tuberculin reactors and 22 tuberculin-negative persons; 1,608 ± 662 pg/ml vs. 372 ± 314 pg/ml; P = 0.00001), which was consistent with previous reports (19). Because the allele *G* in the *MCP-1* promoter increases gene expression (10, 20), we examined plasma MCP-1 levels in patients with different

Table III. The *MCP-1* genotype *GG* is associated with development of pulmonary tuberculosis (Mexican sample)

MCP-1 genotype[a]	TB[b] (*n* = 435)	PPD+[c] (*n* = 334)	TB versus PPD+			PPD−[d] (*n* = 176)	TB versus PPD−		
	n (frequency)	*n (frequency)*	*p-value*	*OR*	*(95% CI)*	*n (frequency)*	*p-value*	*OR*	*(95% CI)*
AA	38 (0.09)	82 (0.25)		1		42 (0.24)		1	
AG	168 (0.39)	161 (0.48)	0.0003	2.3	(1.5–3.5)	88 (0.50)	0.004	2.1	(1.3–3.5)
GG	229 (0.53)	91 (0.27)	0.00001	5.4	(3.4–8.6)	46 (0.26)	0.00001	5.5	(3.2–9.5)

[a]Groups were compared by χ^2 analysis Mantel-Haenszel statistics with genotypes arranged in an ordinal scale.
[b]TB = new cases of pulmonary tuberculosis.
[c]PPD+ = healthy tuberculin reactors.
[d]PPD− = healthy tuberculin-negative persons.

Table IV. The allele *G* of the *MCP-1* promoter region is associated with development of pulmonary tuberculosis (Korean sample)[a]

Allele	TB[b] (258 alleles)	Healthy controls (324 alleles)	TB versus healthy controls
MCP-1 −2518			
A	103 (0.40)	206 (0.64)	
G	155 (0.60)	118 (0.36)	$\chi^2 = 32.28$ $p = 0.00001$

[a]*MCP-1* alleles were typed by amplification fragment length polymorphism analysis. *NOS2A*, *RANTES*, and *MIP-1α* alleles were typed by amplification-created restriction site analysis, as detailed in the methods. Groups were compared by chi-square analysis.
[b]TB = new cases of pulmonary tuberculosis.

MCP-1 genotypes. Carriers of the *GG* genotype had the highest MCP-1 levels (1,976 ± 582 pg/ml), followed by those with the *AG* (1,424 ± 542 pg/ml) and *AA* (1,109 ± 546 pg/ml) genotypes, and these differences were statistically significant (Fig. 1).

IL-12p40 plasma levels were significantly higher in tuberculosis patients from Mexico than in controls (1,270 ± 507 pg/ml vs. 332 ± 284 pg/ml; P = 0.00001). When tuberculosis patients were stratified by *MCP-1* genotypes, levels of IL-12p40 were significantly lower in carriers of the *GG* genotype (1,179 ± 435 pg/ml) than in those with the *AG* (1,348 ± 525 pg/ml) or the *AA* (1,471 ± 508 pg/ml) genotypes (Fig. 2). There was a significant negative correlation between MCP-1 and IL-12p40 levels in persons with the *GG* genotype (correlation coefficient = −0.71; P = 0.00001) but not in those with the *AA* or *AG* genotypes. These findings suggest that overproduction of MCP-1 in patients with the genotype *GG* down-regulated IL-12p40 expression.

MCP-1 inhibits *M. tuberculosis*–stimulated IL-12p40 production by monocytes

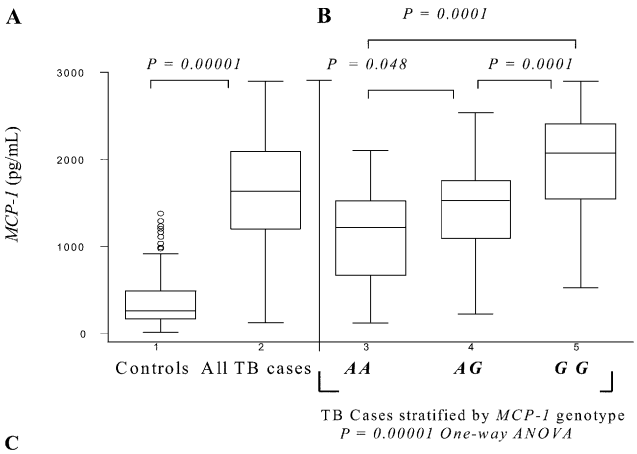
Monocytes are the major sources of MCP-1 and IL-12p40. Because the plasma levels of these two molecules were negatively correlated, we next evaluated production of these cytokines by monocytes from persons with homozygous *GG* and *AA*. We used cells from these because carriers of those genotypes had the highest and lowest plasma levels of MCP-1, respectively, and they represent the extreme phenotypes. Monocytes were stimulated with 5 μg/ml of a sonicate of *M. tuberculosis* H37Rv, because preliminary experiments showed that this concentration induced the highest concentrations of IL-12p40 at 12–72 h of stimulation (unpublished data). Levels of MCP-1 and IL-12p40 in culture supernatants increased with time and were maximal at 48–72 h. MCP-1 levels were significantly higher in homozygous *GG* than in homozygous *AA*, whereas the reverse was true for IL-12p40 levels (Fig. 3). There was a significant negative correlation between levels of MCP-1 and IL-12p40 in supernatants from cells of homozygous *GG* at 12, 24, and 48 h, with correlation coefficients ranging from −0.53 to −0.60 and p-values of 0.01–0.02.

Addition of saturating amounts of anti–MCP-1 antibodies to *M. tuberculosis*–stimulated monocytes from homozygous *GG* significantly increased IL-12p40 levels (5,911 ± 964 pg/ml) compared with monocytes treated with no antibody (3,001 ± 1037 pg/ml) and those treated with isotype control antibodies (3,042 ± 958 pg/ml; Fig. 4). In contrast,

Table V. The *MCP-1* genotype *GG* is associated with development of pulmonary tuberculosis (Korean sample)

MCP-1 genotype[a]	TB[b] (*n* = 129)	Healthy controls (*n* = 162)	TB versus healthy controls		
	n (frequency)	*n* (frequency)	*p-value*	*OR*	*(95% CI)*
AA	20 (0.155)	66 (0.407)		1	
AG	63 (0.488)	74 (0.457)	0.00063	2.8	(1.5–5.1)
GG	46 (0.357)	22 (0.136)	3.2×10^{-8}	6.9	(3.4–14.1)

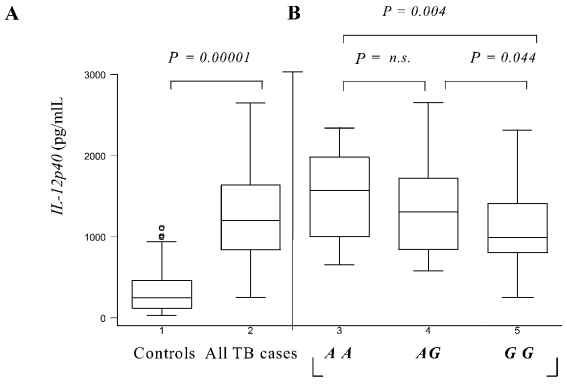
[a]Groups were compared by χ^2 analysis Mantel-Haenszel statistics with genotypes arranged in an ordinal scale.
[b]TB = new cases of pulmonary tuberculosis.



C

	Observations	*Mean*	*SD*	*p value*
Controls	102	372.2	314.14	
TB cases	145	1607.84	661.57	*Cases vs Controls = 0.00001*
	TB cases stratified according to the MCP-1 genotype			
AA	31	1108.97	546.2	*AA vs AG = 0.048* *AA vs GG = 0.0001*
AG	48	1424.44	541.7	*AG vs GG = 0.0001*
GG	66	1975.54	582.17	

Figure 1. Tuberculosis patients with the *MCP-1 GG* genotype have the highest plasma concentrations of MCP-1. Plasma MCP-1 levels were measured by ELISA in 145 tuberculosis patients, 80 healthy tuberculin reactors, and 20 healthy tuberculin-negative controls. There were no differences in MCP-1 levels between the healthy tuberculin reactors and tuberculin-negative persons, so they were combined into a single group (controls). MCP-1 values are shown as medians (horizontal lines), the 25th and 75th percentiles (boxes), and ranges (whiskers). (A) Distribution of plasma MCP-1 values for tuberculosis patients and controls. (B) MCP-1 levels in tuberculosis patients, stratified by *MCP-1* genotype. (C) A summary with values of MCP-1 means and standard deviations is shown. p-values are based on the Bonferroni least significant difference test.



C

	Observations	Mean	SD	p value
Controls	102	331.7	283.8	
TB cases	145	1270.01	507.4	*Cases vs Controls = 0.00001*
TB cases stratified according to the MCP-1 genotype				
AA	31	1470.65	507.73	*AA vs AG = 0.837* *AA vs GG = 0.004*
AG	48	1348.06	525.02	*AG vs GG = 0.044*
GG	66	1178.7	434.6	

Figure 2. Tuberculosis patients with the *MCP-1 GG* genotype have the lowest plasma concentrations of IL-12p40. Plasma IL-12p40 levels were measured by ELISA in 145 tuberculosis patients, 80 healthy tuberculin reactors, and 20 healthy tuberculin-negative controls. There were no differences in IL-12p40 levels between the tuberculin reactors and tuberculin-negative persons, so they were combined into a single group (controls). IL-12p40 values are shown as medians (horizontal lines), the 25th and 75th percentiles (boxes), and ranges (whiskers). (A) Distribution of plasma IL-12p40 levels. (B) IL-12p40 levels in tuberculosis patients, stratified by *MCP-1* genotype. (C) A summary with values of IL-12p40 means and standard deviations is shown. p-values are based on the Bonferroni least significant difference test.

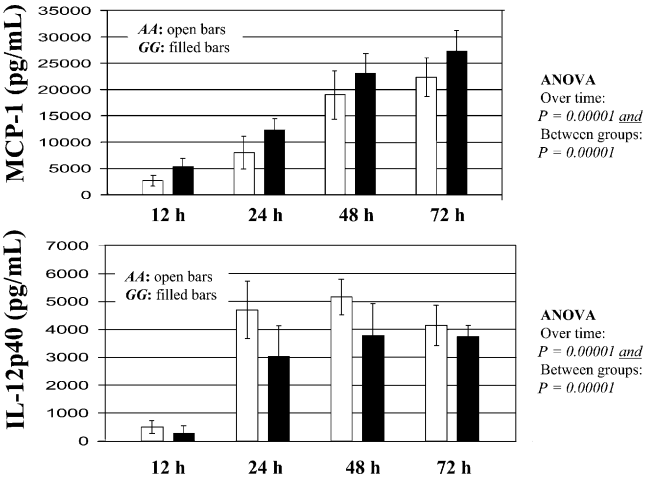


Figure 3. MCP-1 and IL-12p40 concentrations in *M. tuberculosis*–stimulated monocytes. Monocytes from 20 persons with the *AA* genotype and 20 persons with the *GG* genotype were cultured in medium alone or with 5 μg/ml of *M. tuberculosis* sonicate, as outlined in Materials and methods. Supernatants were harvested at the time points indicated, and MCP-1 and IL-12p40 concentrations were measured by ELISA. Mean values and standard deviations are shown for cytokine levels in *M. tuberculosis*–stimulated monocytes. MCP-1 and IL-12p40 were not detected in supernatants of cells cultured in medium alone. In persons with the *GG* genotype, MCP-1 and IL-12p40 concentrations were negatively correlated at 12 h (correlation coefficient = −0.55; P = 0.01), 24 h (correlation coefficient = −0.60; P = 0.01), and 48 h (correlation coefficient = −0.53; P = 0.02). MCP-1 and IL-12p40 levels were not correlated in persons with the *AA* genotype.

anti–MCP-1 did not further increase IL-12p40 production in cells from homozygous *AA*.

To determine whether addition of exogenous MCP-1 would affect *M. tuberculosis*–stimulated production of IL-12p40, we used cells from *MCP-1* homozygous *AA* because they produced high levels of IL-12p40 and relatively low levels of MCP-1 that may not be sufficient to inhibit IL-12p40 production. When we cultured monocytes from these individuals with exogenous MCP-1 before stimulation with *M. tuberculosis,* 2,000 and 4,000 pg/ml reduced MCP-1 concentrations by 24 and 36%, respectively (Fig. 5).

DISCUSSION

We found that the allele *G* of the *MCP-1* promoter–enhancing region is strongly associated with increased odds of developing active pulmonary tuberculosis after infection in Mexicans and Koreans. Persons with the *MCP-1* genotypes *AG* and *GG* were 2.3- and 5.4-fold and 2.8- and 6.9-fold more likely to develop tuberculosis than those with the *AA* genotype in Mexicans and Koreans, respectively. In addition, tuberculosis patients from Mexico carrying the genotype *GG* had the highest plasma levels of MCP-1 and the lowest plasma levels of IL-12p40, and these values were negatively correlated. Furthermore, stimulation of monocytes from normal persons bearing the *GG* genotype with *M. tuberculosis* antigens yielded higher concentrations of MCP-1 and lower concentrations of IL-12p40 than parallel experiments with monocytes from persons of the *AA* genotype. Addition of anti–MCP-1 increased IL-12p40 levels in *M. tuberculosis*–stimulated monocytes from persons of the *GG* genotype, and addition of exogenous MCP-1 reduced IL-12p40 production by *M. tuberculosis*–stimulated monocytes from persons of the *AA* genotype. The sum of these findings suggests that persons bearing the *MCP-1* genotype *GG* are at increased risk for progression of tuberculosis infection to active disease, which is caused by reduced production of IL-12p40 and a depressed Th1 response.

Compared with previous studies (22, 28–34) evaluating genetic factors associated with susceptibility to tuberculosis, this study was distinctive. First, to maximize the likelihood of detecting effects of genetic factors controlling progression to active disease after recent exposure, we selected sputum smear–positive tuberculosis patients with culture-confirmed disease and excluded those with chronic illnesses, including malnutrition, which may predispose to tuberculosis. Second, we selected new tuberculosis cases and excluded those with

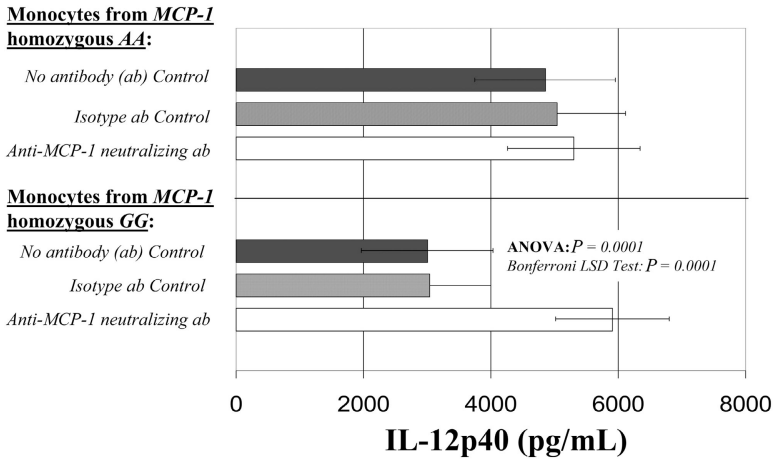


Figure 4. Neutralization of MCP-1 increases IL-12p40 production by *M. tuberculosis*–stimulated monocytes from persons with the *MCP-1 GG* genotype. Monocytes from 20 persons with the genotype *AA* and 20 persons with the genotype *GG* were cultured with 100 μg/ml anti–MCP-1, 100 μg/ml of isotype control antibodies, or no antibodies for 1 h, then stimulated with 5 μg/ml *M. tuberculosis* sonicate, as outlined in Materials and methods. Supernatants were harvested at 24 h, and IL-12p40 concentrations were determined by ELISA. Mean values and standard deviations are shown. IL-12p40 levels in the three groups differed significantly only in persons with the *GG* genotype by one-way ANOVA (P = 0.0001). For persons with the *GG* genotype, values for cells treated with anti–MCP-1 were significantly higher than those of the other two groups (P = 0.0001 using the Bonferroni least significant difference test).

previous episodes of tuberculosis. Third, we chose patients with clinical and epidemiological features that were strongly suggestive of active tuberculosis of short evolution after recent exposure. In the Mexican sample, we selected as controls healthy tuberculin reactors who were not vaccinated with Bacillus Calmette-Guerin (BCG) and had been in recent contact with a tuberculosis case, so we were confident that they were infected with *M. tuberculosis*. We compared allele and genotype frequencies in tuberculosis patients with those in healthy tuberculin reactors and healthy tuberculin-negative persons, allowing us to distinguish susceptibility to progression of infection to disease from susceptibility to tuberculosis infection. In addition, subjects were followed for 3 yr to ensure that they did not develop tuberculosis and, therefore, had protective natural immunity. The *MCP-1* allele *G* was more commonly found in tuberculosis patients compared with healthy tuberculin reactors, demonstrating that this allele increases the likelihood of progression of tuberculosis infection to disease. In contrast, the *MCP-1* allele *G* was equally common in healthy tuberculin reactors and healthy tuberculin-negative persons, indicating that this allele does not increase susceptibility to infection. In summary, by selecting persons with clear phenotypes and by minimizing the effects of nongenetic risk factors, our results provide strong evidence that the *MCP-1* allele *G* and genotype *GG* are associated with increased risk of progression from tuberculosis infection to active disease in Mexicans. This strategy has allowed us to identify a gene influencing expression of disease in other populations. Indeed, we could replicate these findings in Korean tuberculosis cases and healthy controls.

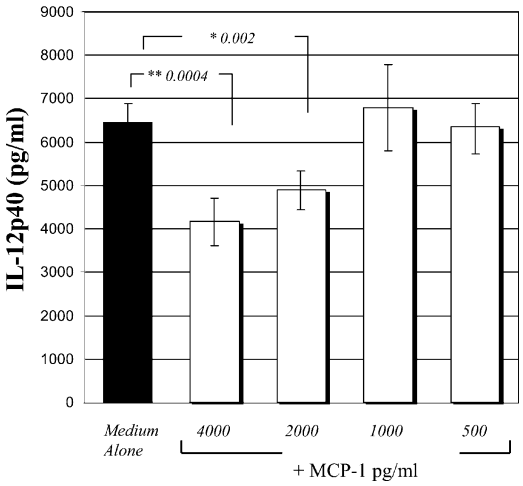


Figure 5. MCP-1 inhibits IL-12p40 production by *M. tuberculosis*–stimulated monocytes. Monocytes from five persons with the *AA* genotype were cultured with 5 μg/ml *M. tuberculosis* sonicate and treated with medium alone or with different concentrations of recombinant human MCP-1, as outlined in Materials and methods. Supernatants were harvested after 24 h, and IL-12p40 was measured by ELISA. Mean values and standard deviations are shown. p-values were corrected for multiple comparisons, and only significant values are shown. IL-12p40 was not detected in supernatants of monocytes treated with medium alone, with or without recombinant MCP-1.

Our results contrast with those of Jamieson et al., who found no association of the *MCP-1* –2518 allele *G* with susceptibility to tuberculosis in a study of cases and pseudocontrols derived from 92 families in Brazil (3). Our study may have yielded positive results because large case-control studies of unrelated persons have an inherently higher power to detect genes controlling the expression of complex traits than

small, family-based studies (21). Discrepant results may have also arisen from differences in the study design. We have conducted case-control studies of unrelated individuals in which genotype and exposure or genotypes at unlinked loci occur independently, whereas Jamieson et al. used cases and pseudocontrols derived from nuclear families (3), a design where those critical features are lost (21). This severely decreases the power to detect genes involved in the expression of complex multifactorial diseases because adjustment for correlations and analytical methods that rely on conditional probabilities are required for the analysis of case-pseudocontrol studies (21). Alternatively, differences in the characteristics of the populations studied may explain our discrepancies. Genomic screening studies have identified several markers linked to tuberculosis susceptibility that differ from population to population (22, 23). Population-dependent variations in the frequency of susceptibility alleles and in the strength of linkage disequilibrium between markers and differences in environmental conditions and lifestyles may explain these apparently discrepant results (24–27). Likewise, in candidate gene studies, the *natural resistance–associated macrophage protein 1*, *vitamin D receptor*, *IL-10* genes, and the *IL-1* cluster of genes have been associated with susceptibility to tuberculosis in some ethnicities but not in others (28–34), supporting the notion that the influence of individual genes may vary in populations that differ in susceptibility allele frequencies or the composition of other susceptibility genes or in environmental factors that alone or in interaction induce the expression of disease susceptibility (24–27). Future studies with sufficient power may identify *MCP-1* as a gene with important main or interactive contributions in susceptibility to developing tuberculosis. We anticipate that our results will have a substantial impact in the field because of the very high frequency of the predisposing *MCP-1* allele *G* and the considerably increased odds of developing disease in carriers of this allele in two populations with different ethnicities.

Our data suggest that very high levels of MCP-1 inhibit IL-12p40 production in carriers of the *MCP-1* genotype *GG,* perhaps adversely affecting the immune response to *M. tuberculosis* infection. Indeed, only in tuberculosis cases and in cultures of cells from carriers of the *MCP-1* genotype *GG* did we observe that IL-12p40 levels were negatively correlated with corresponding MCP-1 concentrations. Our findings suggest that IL-12p40 may be down-regulated by MCP-1 concentrations only when they are above a certain threshold. Only saturating amounts of anti–MCP-1–neutralizing antibodies restored IL-12p40 production by *M. tuberculosis*–stimulated monocytes from individuals of the *GG* genotype, and only the addition of very high concentrations of MCP-1, equivalent to those in plasma of tuberculosis cases carrying the genotype *GG*, inhibited *M. tuberculosis*–induced IL-12p40 production by monocytes from individuals of the *AA* genotype (Fig. 5). Our in vitro observations are consistent with previous in vitro studies in human monocytes (35) and murine dendritic cells, demonstrating that MCP-1 inhibits IL-12 production (36, 37). Moreover, only those transgenic mice producing very high levels of MCP-1 had increased susceptibility to disease from intracellular pathogens, including *M. tuberculosis* (38).

The IL-12/IL-23/IFN-γ axis plays a pivotal role in resistance to intracellular pathogens, including *M. tuberculosis* (39–46). IL-12p40 is a component of IL-12 and IL-23 and is required for their binding to the IL-12 receptor β1 subunit (47). Thus, inhibition of IL-12p40 by MCP-1 in *MCP-1* homozygous *GG* could contribute to development of tuberculosis. In agreement with this concept, individuals carrying mutations in the *IL-12p40* and *IL-12 receptor β1* genes have increased susceptibility to mycobacterial infection (48–50).

In summary, we found that the *MCP-1* –2518 *G* allele has a dose effect on the likelihood of progression of tuberculosis infection to disease in Mexicans and Koreans, and that Mexicans and Koreans with the *GG* genotype were 5.4- and 6.9-fold more likely, respectively, to develop tuberculosis than those with the *AA* genotype. Correlation of MCP-1 and IL-12p40 plasma levels in Mexicans, as well as in vitro experiments with MCP-1 and IL-12p40, suggests that persons with the genotype *GG* produce very high concentrations of MCP-1, which inhibits production of IL-12p40 and increases the odds that *M. tuberculosis* infection will progress to disease.

MATERIALS AND METHODS

Sample size calculation

Preliminary data from 40 tuberculosis cases and 40 controls from Mexico showed that the frequencies of the *MCP-1* allele *G* were 0.75 and 0.45, respectively, in tuberculosis cases and controls. Based on these data, we calculated that a sample size of 251 tuberculosis cases and 251 controls would provide 90% power to detect an OR of 2 with a two-sided α of 0.01. To ensure adequate power after correction for population stratification and multiple comparisons, we enrolled 445 tuberculosis patients and 518 controls.

Subjects

We conducted unmatched case-control studies in Mexico and Korea.

Mexican sample. Tuberculosis patients and controls were Mexican adults of Mestizo ethnicity (18–50 yr old) recruited in Mexico D.F. as part of the World Health Organization's DOTS community surveillance program for early detection of new tuberculosis cases (13). All subjects had negative serologic tests for HIV infection, were of similar socioeconomic statuses, and were unrelated to the third generation, as determined by a questionnaire. Persons with a prior history of tuberculosis, cancer, organ transplantation, primary immunodeficiency, therapy with immunosuppressive drugs such as corticosteroids, asthma, autoimmune or endocrine disorders, or chronic cardiopulmonary, hepatic or renal disease were excluded. The BMI for each subject was determined, based on self-reported weight, before disease in the case of tuberculosis patients, and height was measured by a nurse. Persons with a BMI <18.5 kg/m^2 were considered to be malnourished (51–53) and were excluded.

From April 1999 through July 2004, 445 tuberculosis cases and 518 healthy controls were enrolled in the study. 20 tuberculosis patients (4%) and 25 controls (5%) declined to participate in the study. All subjects provided informed consent under protocols approved by the institutional review boards of the Dana-Farber Cancer Institute and Mexican Instituto Nacional de Ciencias Medicas y Nutricion "Salvador Zubiran."

Tuberculosis cases had symptoms (weight loss >10 kg, cough, fever, night sweats for >1 mo, or cervical or axillary lymphadenopathy) and chest

radiographic findings consistent with recent pulmonary tuberculosis, a positive sputum acid-fast smear and culture confirmed for *M. tuberculosis*, and a history of substantial exposure to tuberculosis in the preceding 8 mo. Patients with predominant upper lobe infiltrates, cavitary disease, military tuberculosis, or parenquimal or pleural fibrosis on chest radiographs were excluded to increase the chances of recruiting new cases of tuberculosis (54, 55). Patients with pleural effusions on chest radiographs were also excluded to avoid misclassification, because tuberculosis pleuritis could also be a manifestation of remote infection (56).

Controls were healthy adults who had recent contact with a tuberculosis patient, and most were neighbors or co-workers of the tuberculosis cases. A tuberculin skin test was administered to all controls, using the Mantoux method to deliver 5 tuberculin U of purified protein derivative RT/23 (Statens Serum Institut) intradermally (57). The diameter of induration was measured 48 h after inoculation. Tuberculin-negative persons were retested 1 wk later to confirm the result. Controls were stratified into tuberculin-positive and -negative groups. All tuberculin reactor controls had tuberculin reactions of ≥10 mm, have had at least three negative sputum smears for acid-fast bacilli, and normal chest radiographs. They were followed for at least 3 yr and did not develop tuberculosis. None of these individuals received isoniazid, consistent with standard medical practice in Mexico. We carefully selected tuberculin reactor controls that were not vaccinated with *M. bovis* BCG as they had no scars suggestive of BCG vaccination (58) and denied a history of having BCG vaccination. Tuberculin-negative controls had two consecutive tuberculin tests that showed <5 mm of induration. Controls whose tuberculin skin tests showed 5–10 mm of induration were excluded from the study.

Korean sample. Tuberculosis patients and controls were Korean adults (17–78 yr old) recruited from Chungnam National University Hospital, Konyang University Hospital, and Bok-syp-ja Clinic, all of which are located in Daejeon, South Korea. Unrelated, healthy blood donors were recruited as controls. Patients with tuberculosis were included after the diagnosis was made by medical, biochemical, and radiological assessment, microscopic examination of sputum smear using Ziehl-Neelsen staining, and culture of *M. tuberculosis* from sputum. All subjects had negative serologic tests for HIV infection and had no serious illnesses other than tuberculosis. DNA samples from 129 tuberculosis cases and 162 healthy controls were used for SNP analysis. The study was approved by the Bioethics Committee of Chungnam University Hospital's review board overseeing studies on samples from human subjects, and all participants gave written consent.

Blood samples

Blood samples (~1 ml) were stored at −20°C, thawed in batches of 50, and centrifuged to obtain cellular pellets. Genomic DNA was isolated from these pellets with DNA extraction kits (QIAGEN). Some aliquots of plasma (~0.5 ml) were also stored at −20°C and used for ELISA tests. Blood samples from Koreans were provided by S.-S. Jung, J.-W. Son, and Y.-J. Lim (Chungnam National University Hospital, Daejeon, South Korea).

SNP analysis

Many SNPs are present in the *MCP-1*, *NOS2A*, *RANTES*, and *MIP-1α* genes. We studied SNPs that alter expression of the gene or were associated with disease (10–12). These SNPs were genotyped in duplicate discrepancies solved by sequencing. There were no discrepancies in typing results of MCP-1. We observed discrepant data in <5% of the cases for the RANTES SNP, which were resolved by sequencing. The region containing the –2518 *G* to *A* transition in the *MCP-1* promoter region (10) was amplified from 100 ng of genomic DNA using the forward primer (5′-GCTCCGGGCCCAGTATCT-3′) and reverse primer (5′-ACAGGGAAGGTGAAGGGTATGA-3′) and a Hot Start PCR (Applied Biosystems). Restriction fragment length after PCR was used for the detection of *MCP-1* alleles. The allele *G* creates a *PvuII* restriction site yielding two fragments of 182 and 54 bp, respectively. The allele *A* was identified by the presence of a 236-bp undigested fragment. To confirm our results in MCP-1 gene analysis, we sequenced 50 randomly selected cases and controls, respectively. The SNPs in the *NOS2A G −954 C* (11), *RANTES G −471 A*, and *MIP-1α C+459 T* (12) transitions were typed by amplification-created restriction sites, as previously described.

Studies of MCP-1 and IL-12p40

Plasma was obtained from a convenience sample of 145 tuberculosis cases, 80 tuberculin reactors, and 22 tuberculin-negative controls from Mexico. These samples were obtained before initiating therapy. MCP-1 and IL-12 p40 levels were measured by ELISA (BD Biosciences).

Buffy coats or leukopacks were obtained at the Dana-Farber Cancer Institute blood bank from healthy donors (20 *MCP-1* homozygous *GG* and 25 homozygous *AA*). PBMCs were obtained by Ficoll-gradient centrifugation. 10^6 PBMCs were used to isolate genomic DNA. The remaining PBMCs were frozen in FCS and 5% DMSO in liquid nitrogen until used. Monocyte-enriched preparations were obtained from PBMC by NycoPrep (Axis-Shield) gradient centrifugation. These contained 75–90% monocytes, as assessed by flow cytometry with anti-CD14– and Giemsa-stained cytocentrifuge preparations. Based on these percentages, the number of monocytes included in the experiments outlined in the next two paragraphs was calculated.

In some experiments, 7×10^6 monocytes/ml were cultured in triplicate in 48-well plates in a final volume of 0.5 ml of complete RPMI 1640, with or without 5 μg/ml *M. tuberculosis* H37Rv sonicate for 72 h. Supernatants were collected at time points ranging from 12–72 h, and IL-12p40 and MCP-1 levels were measured in supernatants by ELISA.

In experiments involving the addition of antibodies or recombinant human MCP-1 (R&D Systems), monocytes were cultured at 7×10^6 cells/ml in complete RPMI 1640 with 10% FCS in petriPERM hydrophobic Petri dishes (Vivascience AG) for 1 h, with or without different concentrations of recombinant human MCP-1, anti–MCP-1–neutralizing antibodies, or isotype control antibodies (both from BD Biosciences). Cells were then washed three times with RPMI 1640, resuspended, and plated in 48-well plates at 7×10^6 cells/ml in 0.5 ml of complete RPMI 1640 and 10% FCS with 5 μg/ml H37Rv sonicate, with or without recombinant human MCP-1, anti–MCP-1, or isotype control antibodies, for 24 h. Supernatants were collected and stored at −20°C until IL-12p40 concentrations were measured.

Statistical analyses

Statistical analysis was done with Intercooled STATA9 software (Stata Corporation). Hardy-Weinberg equilibrium was calculated using χ^2 tests for $n(n + 1)/2$ degrees of freedom, where n is the number of alleles in the polymorphism tested (59). Expected genotype proportions were obtained using allele frequencies observed in the controls and the binomial equation.

Associations of alleles with disease were analyzed using 2×2 contingency tables, two-sided χ^2, or Fisher's exact tests, as appropriate (60). For the analysis of allele associations with disease in the Mexican sample, χ^2 values were corrected for population stratification, primarily to control for differences in levels of admixture between cases and controls, by dividing these χ^2 values by an estimated value of λ (15). λ was calculated as the mean of χ^2 values from comparison of the allele frequencies of 30 SNPs located across the genome (15, 16) that were not in linkage disequilibrium (Table S1). The resulting χ^2 values were further adjusted according to the number of comparisons using the Bonferroni correction.

We used multiple 2×2 tables, with genotypes arranged in an ordinal scale, and χ^2 Mantel-Haenszel statistics to test for genotype association with disease. To test for additive effects we used the score test for trend of the ORs.

Differences in MCP-1 and IL-12p40 levels in plasma between cases and controls and in some in vitro studies were determined using unpaired Student's *t* tests (with the Bonferroni correction) as appropriate. One-way analysis of variance (ANOVA), followed by the Bonferroni least significant difference test for multiple comparisons, was used to analyze plasma cytokine levels and to examine in vitro IL-12p40 levels in samples treated with anti–MCP-1 antibodies. Pearson's and Spearman's tests were used to evaluate correlations. To examine mean cytokine levels in culture supernatants over time in homozygous *GG* versus *AA*, we used two-group repeated measures ANOVA with subjects nested in groups. Before running ANO-

VAs we confirmed that normality (normal distribution of the data) and homocedasticity (the variances were homogeneous) assumptions were not violated, using Shapiro-Wilk and Bartlett's tests, respectively.

Online supplemental material

Table S1 lists the SNP typed as genomic controls, frequency of alleles at each loci, and χ^2 values resulting from comparisons of allele frequencies in Mexican cases and controls. At the end of the table the value λ, calculated as outlined in Statistical analyses, is presented. Table S2 shows the demographic and clinical features of Korean cases and controls. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20050126/DC1.

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