Host gene-encoded severe lung TB: from genes to the potential pathways

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We are reporting that the two-locus genotype − 2518 macrophage chemotactant protein (MCP)-1 GG and − 1607 matrix metalloproteinase (MMP)-1 2G/2G promotes the expression of hyperinflammation in response to Mycobacterium tuberculosis infection, inducing extensive tissue damage and severe tuberculosis (TB) disease. Carriers of this two-locus genotype have a 13-fold higher chance of developing severe disease and 6.5-fold higher chance of developing permanent lesions, and a 3.864-fold higher chance of delayed response to first-line standardized treatment than carriers of any other relevant combination of genotypes at those two loci. Thus, these persons have an increased likelihood of poor health-related quality of life and of transmitting M. tuberculosis to other members of the community. In addition, through the analysis of human lung tissues, serum/plasma and in vitro experiments, including in vitro infections of THP-1 cells with M. tuberculosis and microarray analysis, we determined that this hyperinflammation state is potentially driven by the MCP-1/MMP-1/PAR-1 pathway. Hence, we are providing markers for the identification of TB cases that may develop severe pulmonary disease and delayed response to treatment, and are providing the basis for development of novel host-targeted clinical interventions to ameliorate the severity of pulmonary TB.

Keywords: tuberculosis; genetics; disease severity; MCP-1; MMP-1

INTRODUCTION

Knowledge of genetic predictors of disease severity may be useful as markers for detection of tuberculosis (TB) patients at risk of developing a severe TB disease. Revealing the mechanisms of severe disease in these patients may provide molecular targets for novel clinical interventions. It is well established that functional genetic polymorphisms in specific molecules of the immune/inflammatory response increase the likelihood of progression from Mycobacterium tuberculosis infection to active pulmonary TB.1–7 Active TB may evolve into severe lung disease, or even to extra-pulmonary disease if left untreated.1–7 We propose that expression of severe pulmonary TB disease is genetically controlled.

Human host-encoded susceptibility to expression of severe pulmonary TB may involve: (i) the spread of infection beyond a critical number of cells because of a failing immune response against this challenge4,7 and (ii) an excessive inflammatory response (hyperinflammatory response), resulting in extensive inflammation-caused tissue damage.5 These mechanisms are not necessarily mutually exclusive. We previously reported that a functional single-nucleotide polymorphism (SNP) located in position − 2518 of the enhancing promoter region of the macrophage chemoattractant protein (MCP)-1 gene (the genotype GG) is associated with the expression of active TB disease in humans.1 Our ex vivo and in vitro studies indicated that increased MCP-1 production in response to infection is driving the expression of disease in carriers of that susceptibility genotype.1,2 MCP-1 is a potent chemokontact of macrophages that, in excess, downregulates IL-12p40 production,8–10 and upregulates matrix metalloproteinase (MMP)-1 production by these cells in response to M. tuberculosis antigens, in vivo and in vitro.2 IL-12p40 is a subunit of two cytokines that are essential in the development of cellular immunity against TB, IL-12 and IL-23.3 On the other hand, human MMP-1 is a potent collagenase, and collagen is an essential component of the granulomatous matrix over which cells (such as macrophages, T-cells and so on) become organized to form granulomas and also a very important component of the lung matrix.2,11 Thus, MMP-1 may be involved in liquefaction of mature granulomas. MMP-1 may also oppose to granuloma formation and maturation. MCP-1, MMP-1 and MMP-9 may contribute to non-granulomatous damage of lung tissue in patients affected by TB. Given that granulomas may serve to enclose infected cells and free bacteria in a confined environment, we and other investigators hypothesize that excessive MMP-1 levels may promote the spread of infection and inflammation.2,12–17

In search for genetic modifiers of the − 2518 MCP-1 genotype GG, we reported that Hispanics with Amerindian ancestry carrying the two-locus genotype − 2518 MCP-1 GG − 1607 MMP-1 2G/2G are at a significant increased chance of progression from M. tuberculosis infection to active TB disease.2 Of note, the − 2518 MCP-1 allele G and the − 1607 MMP-1 allele 2G creates a
transcription activator-like effector binding site for PREP1/PRX2 transcription factor complexes\textsuperscript{18,19} and increases the rate of transcription of the MCP-1 gene, which is active in TB.\textsuperscript{1,2,20} Likewise, the –1607 MMP-1 allele 2G, which consists of the insertion of a guanine at position –1607 (the allele 2G), creates an Ets-1 transcription factor binding site\textsuperscript{12,22} and enhances expression of the gene in normal and tumor cells and in active TB.\textsuperscript{21,22}

Whether the two-locus susceptibility genotype –2518 MCP-1 GG and –1607 MMP-1 2G/2G may also influence the expression of a more severe disease is unknown. Thus, in this study we address this question. We also conducted \textit{ex vivo} and in vitro experiments to unveil potential mechanisms of disease severity in carriers of this two-locus susceptibility genotype. Data from this study could guide the development of new therapeutic approaches to overcome host-encoded deleterious hyperinflammatory response to \textit{M. tuberculosis} infection and expression of severe pulmonary TB. Here we are also demonstrating that excessive MMP-1 may potentiate the MCP-1 and \textit{M. tuberculosis}-induced inflammatory response through activation of protease-activated receptor (PAR)-1, and thereby increases the likelihood of developing a severe TB disease.

RESULTS
Demographic and clinical features of TB cases studied
We recruited \textit{de novo} 224 patients with TB that met the enrollment criteria and were tightly followed through the directly observed therapy program, ensuring adherence to therapy. We did not see differences in the proportion of males and females, nor in the mean age between groups (Table 1). None of the patients self-reported consumption of alcohol or tobacco. All of them were of low socioeconomic status and Peruvian Mestizo to Spaniards. All of the patients were recruited in similar geographical areas, where they had lived for more than 2 years, making it more likely that they were infected by the same bacterial strains circulating in the region. They were all Bacille Calmette Guerin-vaccinated, and had a disease confined to the lungs of not more than 3 months of evolution. We did not see differences in duration of disease (the length of time that passed since the first symptom was noticed by the patient to the time of sampling) (Table 1). To test for differences in the admixture of TB cases stratified according to genotypes, we genotyped 42 genomic controls

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| Two-locus genotypes | Group 1: A/– 1G/– | Group 2: A/– 2G/2G | Group 3: GG 1G/– | Group 4: GG 2G/2G | Statistics |
|---------------------|-------------------|-------------------|-----------------|-----------------|------------|
| TB cases per genotype n (%) (total n = 224) | 51 (23%) | 59 (26%) | 43 (19%) | 71 (32%) | n.s.\textsuperscript{a} |
| Mean age ± s.d. | 33.9 ± 10.8 | 31.63 ± 10.9 | 28.95 ± 9.3 | 29.7 ± 10.06 | n.s.\textsuperscript{a} |
| Women n (%) | 23 (45%) | 27 (46%) | 20 (47%) | 31 (44%) | n.s.\textsuperscript{a} |
| Duration of disease (days) | 63 ± 20 | 64 ± 20 | 65 ± 22 | 64 ± 24 | n.s.\textsuperscript{a} |
| BMI ≤ the median n (%) Median = 21.21 | 19 (37.3) | 26 (44.06) | 22 (51.2) | 45 (63.4) | $P = 0.026$\textsuperscript{a} |
| Severity score > median n (%) median = 44 | 14 (27.5) | 20 (33.9) | 8 (18.6) | 59 (83) | $P < 0.001$\textsuperscript{a} |

Abbreviations: BMI, body mass index; n, number of cases; n.s., not significant; TB, tuberculosis. Group 1 = genotypes AA 1G/1G, AA 1G/2G, AG 1G/1G and AG 1G/2G. Group 2 = genotypes AA 2G/2G and AG 2G/2G. Group 3 = genotypes GG 1G/1G and GG 1G/2G. Group 4 = genotypes GG 2G/2G. *One-way ANOVA was used to test for significant differences. $\chi^2$-test was used to test for significant differences.
MMP-1 2G/2G express a more severe TB disease than carriers of any other relevant combination of genotypes at these two loci.

TB patients carrying the two-locus genotype – 2518 MCP-1 GG and – 1607 MMP-1 2G/2G experienced a delayed response to standardized treatment and extensive fibrosis surrounding cavities and bronchiectasis at the end of the treatment. Most of our TB cases observed a sputum smear test conversion from positive to negative at more than 3 months (mean ± s.d. = 3.7 ± 0.6) and a delayed response to treatment according to the World Health Organization (Table 5). We asked whether disease severity, which reflects excessive tissue damage, will influence the response to the standardized TB treatment. Most of our TB cases observed a sputum smear test conversion from positive to negative at the second month of treatment. In contrast, we observed that 29.6% of TB patients carrying the two-locus genotype – 2518 MCP-1 GG and – 1607 MMP-1 2G/2G experienced a significant delayed response to treatment (Table 5). The test for homogeneity of the odds and the score test for trend in odds indicate the presence of a dose–response relationship, were carrying the genotypes – 2518 MCP-1 GG and the – 1607 MMP-1 2G/2G, conferred the highest odds of a sputum test conversion from positive to negative at more than 3 months (mean ± s.d. = 3.7 ± 0.6) and a delayed response to treatment according to the World Health Organization (Table 5).

At the end of the treatment, a greater proportion of TB cases, all carrying the two-locus genotype – 2518 MCP-1 GG and – 1607 MMP-1 2G/2G, had more than 2/3 of lung parenchyma compromised by fibrotic lesions than carriers of any other genotypes, which was reflected by the presence of dyspnea (difficulty breathing). In all, 33 out of 71 TB cases carrying the susceptibility two-locus genotype (83%) versus 18 out of 153 TB cases (37.8%) in carriers of any other combination of genotypes expressed those permanent lesions (odds ratio (OR) = 6.5, 95% confidence interval (CI) = 3.14–13.6, \( \chi^2 = 33.23, P < 0.00001 \)). Lung fibrosis was mainly observed surrounding cavities and bronchiectasis.

Carriers of the two-locus genotype – 2518 MCP-1 GG and – 1607 MMP-1 2G/2G express the highest levels of MMP-9 Using immunohistochemistry (IHC), we assessed the levels of MCP-1, MMP-1 and MMP-9 expression by alveolar macrophages in lung tissue from those six TB cases that required surgery to remove damaged tissue. MMP-9 was tested because, in the Zebra fish model of TB, MMP-9 was shown to drive the recruitment of monocytes to the granulomatous inflammatory foci, where these cells were exposed and infected with Mycobacterium marinum, resulting in a significant increase in the burden of infected cells. Because of the extensive lung damage observed, we focused our analysis on granulomas located in areas of lung parenchyma surrounding extensive necrotic areas. We observed several macrophages and other cells (that is, lung epithelial cells) expressing copious amounts of MCP-1, MMP-1 and MMP-9 in these tissues (Figure 1). The disease tissue slices showed multiple dark purple cells, which could be interpreted as macrophages (red for CD68) expressing MCP-1, MMP-1 and MMP-9 (blue for each factor). We could not assess whether those levels observed in lung tissues were higher in carriers of the – 2518 MCP-1 GG and – 1607 MMP-1 2G/2G than in carriers of any other combination of genotypes at these two loci because of the lack of tissues from the latter groups. Hence, to further determine whether genotypes will correlate with phenotypes in these TB cases, we measured the serum levels of MCP-1 and plasma levels of MMP-1 and MMP-9 (Figure 2).

Thirty randomly selected cases per each of the four relevant two-locus genotypes – 2518 MCP-1 and – 1607 MMP-1 and 20 healthy controls (only as reference) were assessed. MMP-9 was significantly overexpressed in TB patients carrying the two-locus

### Table 2. Severity score

| Clinical signs and symptoms | Score if present |
|-----------------------------|------------------|
| Cough                       | 10               |
| Dyspnea                     | 10               |
| Night sweats                | 10               |
| Haemoptysis                 |                  |
| At least one episode        | 10               |
| More than one episode       | 20               |
| Chest pain                  | 10               |
| Decreased body mass index (BMI) in adults | |
| BMI < 18.5 to 17            | 2                |
| BMI < 18.5 to 17            | 4                |
| BMI < 18.5 to 17            | 6                |
| BMI < 18.5 to 17            | 10               |
| Low BMI for age percentile in children | |
| BMI < 50th but > 25th percentile for age | 2              |
| BMI in the 25th to no more than 10th percentile | 4            |
| BMI in the 10th to more than 5th percentile | 6            |
| BMI below the 5th percentile | 10               |
| Anemia                      | 10               |
| Fever                       | 10               |
| Signs at lung auscultation  | 10               |
| Extra-thoracic involvement  | 10               |
| Chest X-ray assessment      | 20               |

### Table 3. Contribution of age, gender and – 2518 MCP-1 – 1607 MMP-1 two-locus genotypes in TB disease severity

| Dependent variables | OR | S.e. | \( Z \) | Prob>|\( Z \)| | 95% CI |
|---------------------|----|------|--------|----------------|--------|
| Age                 | 0.998 | 0.0154 | –0.10 | 0.921 | 0.969–1.029 |
| Gender (female)     | 0.667 | 0.215 | –1.26 | 0.209 | 0.355–1.254 |
| Group 2: A/ – 2G/2G | 1.358 | 0.571 | 0.73  | 0.467 | 0.595–3.095 |
| Group 3: GG         | 0.6  | 0.306 | –1.00 | 0.317 | 0.222–1.63  |
| 1G/1G               |      |       |       |       |          |
| Group 4: GG         | 13.13 | 5.941 | 5.69  | 0.0001 | 5.41–31.88 |

Pearson’s goodness-of-fit: \( \chi^2 = 151.44, P = 0.13 \)

Abbreviations: CI, confidence interval; LR, likelihood ratio; OR, odds ratio. Genotype A/- 1G/- = genotypes AA 1G/1G or AA 1G/2G, AG 1G/1G or AG 1G/2G. Genotype A/- 2G/2G = genotypes AA 2G/2G, AG 2G/2G, GG 2G/2G. Genotype GG 1G/- = genotypes GG 1G/1G, GG 1G/2G. Group 1 was used as reference. Number of observations = 224. LR \( \chi^2 (5) = 68.62, P < 0.000001 \).
genotype −2518 MCP-1 GG and −1607 MMP-1 2G/2G, as compared with the levels of MMP-9 in carriers of any other combination of genotypes at these two loci (Figure 2). A less significant increase in MCP-1 and MMP-1 was also observed in carriers of the two-locus genotype −2518 MCP-1 GG and −1607 MMP-1 2G/2G, when compared with the levels of these factors in carriers of other combinations of genotypes at these two loci (Figure 2). Controls had only normal levels of MMP-9 (24.760 ± 13.050 ng ml⁻¹) and MMP-1 (6.12 ± 2.76 ng ml⁻¹) in plasma and MCP-1 (563 ± 328 pg ml⁻¹) in serum. Furthermore,

Table 4. Mean score value associations with the −2518 MCP-1 and −1607 MMP-1 two-locus genotypes

| Two-locus genotypes | Cases (n) | Score mean ± s.d. | Rank sum | Bonferroni LSD |
|---------------------|----------|-------------------|----------|---------------|
| Group 1: A/− 1G/−   | 51       | 39.45 ± 11.97     | 4047     | G1 versus G2  P = n.s. |
| Group 2: A/− 2G/2G  | 59       | 43.96 ± 12.1      | 6004.5   | G2 versus G3  P = n.s. |
| Group 3: GG 1G/−    | 43       | 39.81 ± 15.21     | 3439     | G3 versus G4  P < 0.0001 |
| Group 4: GG 2G/2G   | 71       | 61.58 ± 17.3      | 11709.5  |               |

Kruskal–Wallis test χ² with ties = 72.689, df. = 3, P = 0.001

Abbreviations: d.f., degrees of freedom; G1, group 1; G2, group 2; G3, group 3; G4, group 4; LSD, least significant difference; n, number of cases; n.s., not significant. Genotype A/− 1G/− = genotypes AA 1G/1G or AA 1G/2G, AG 1G/1G or AG 1G/2G. Genotype A/− 2G/2G = genotypes AA 2G/2G, AG 2G/2G.

Table 5. Associations of the −2518 MCP-1 and −1607 MMP-1 two-locus genotypes and sputum conversion at more than 3 months

| Two-locus genotypes | Cases n (%) | OR | Prob > χ² | 95% CI |
|---------------------|-------------|----|-----------|-------|
| Group 1: A/− 1G/−   | 5 (9.8)     | 1  | —         | —     |
| Group 2: A/− 2G/2G  | 9 (15.3%)   | 1.4| 0.57      | 0.44–4.5 |
| Group 3: GG 1G/−    | 6 (13.9%)   | 1.492| 0.54 | 0.41–5.33 |
| Group 4: GG 2G/2G   | 21 (29.6%)  | 3.864| 0.009 | 1.3–11.5 |

χ² = 10.41, df. = 3; Prob > χ² = 0.0154 χ² = 8.61, df. = 1; Prob > χ² = 0.0034

Test of homogeneity (equal odds): score test for trend of odds

Abbreviations: CI, confidence interval; df., degrees of freedom; n, number of cases showing sputum conversion > 3 months; OR, odds ratio. Genotype A/− 1G/− = genotypes AA 1G/1G or AA 1G/2G, AG 1G/1G or AG 1G/2G. Genotype A/− 2G/2G = genotypes AA 2G/2G, AG 2G/2G. Genotype GG 1G/− = genotypes GG 1G/1G, GG 1G/2G.

Figure 1. TB patients carrying the two-locus genotype −2518 MCP-1GG and −1607 MMP-1 2G/2G express MCP-1, MMP-1 and MMP-9. We used double IHC analysis of MCP-1, MMP-1 or MMP-9 and CD68 in paraffin-embedded diseased lung from six TB patients who underwent surgery to remove damaged tissue. We are showing representative data. (a) Negative control (irrelevant antibodies isotype control). (b) MCP-1 (blue) and CD68 (red). (c) MMP-1 (blue) and CD68 (light red) (d) MMP-9 (blue) and CD68 (light red). Images were acquired at ×200 total magnification. IHC analysis shows granulomas with CD68-positive cells (macrophages) expressing copious amounts of MCP-1, MMP-1 and MMP-9.
we obtained significant positive correlations between MCP-1 levels (Spearman’s Rho = 0.91; P < 0.00001) or MMP-1 levels (Spearman’s Rho = 0.93; P < 0.00001) or MMP-9 levels (Spearman’s Rho = 0.95; P < 0.00001) and the scores in TB cases carrying the two-locus genotype. 1 = two-locus genotype A'/1G'/A'/1G'; 2 = two-locus genotype A'/2G/2G'; 3 = two-locus genotype GG 1G'/A'; and 4 = two-locus genotype GG 2G/2G'.

In vitro studies revealed the underlying mechanisms of increased MMP-9

Given that we observed the highest plasma levels of MMP-9 in TB patients carrying the two-locus genotype — 2518 MCP-1 GG and — 1607 MMP-1 2G/2G — we reasoned that this might result from more complex preceding molecular events, where MCP-1 and MMP-1 were involved. We used in vitro exposure of human THP-1 monocytic cells to sonicated H37Rv M. tuberculosis to model the exposure of massively recruited monocytes to the lung inflammatory foci during TB-induced inflammatory response.28–31 We observed that exposure of THP-1 cells to sonicated H37Rv M. tuberculosis for 24 h caused a significant increase in the expression of MCP-1, MMP-1 and MMP-9 (Figure 3). In contrast, this stimulus inhibited the expression of tissue inhibitor of metalloprotease (TIMP)-1, -2, -3 and -4 (Figure 3). To test the notion that MCP-1 increases MMP-9 production, we activated THP-1 cells with sonicated H37Rv M. tuberculosis and inhibited CCR2 with the compound RS504393 (Figure 3, panel 1). MCP-1 effects are likely inhibited by this compound because CCR2 is the only receptor for MCP-1.32 MMP-9 mRNA expression in response to the sonicated H37Rv M. tuberculosis stimuli was significantly decreased by this CCR2-inhibiting compound (Figure 3, panel 1). MCP-1 and MMP-1 mRNA were also significantly downregulated by this CCR2 inhibitor (Figure 3, panel 1). Although the expression of natural inhibitors of MMPs (TIMP-1, -2, -3 and -4) was poor, as compared with that of MMP-1 and MMP-9, RS504393 appeared to slightly counteract the inhibitory effect of the sonicated H37Rv M. tuberculosis exposure on TIMP expression (Figure 3, panel 1).

We next investigated whether inhibition of MMP-1 would provide similar results. Using the 4-Aminobenzoyl-Gly-Pro-D-Leu-D-Ala hydroxamic peptide (at 1 mM concentration) to inhibit MMP-1 functions, we could also downregulate MMP-9 (Figure 3, panel 2). Although MCP-1 was not significantly downregulated, MMP-1 expression was significantly inhibited by addition of the 4-Aminobenzoyl-Gly-Pro-D-Leu-D-Ala hydroxamic peptide to cultures of THP-1 cells activated with sonicated H37Rv M. tuberculosis (Figure 3, panel 2). TIMP expression was not significantly modulated by this MMP-1 inhibitor (Figure 3, panel 2).

MMP-1 may activate PAR-1 expression on monocytes/macrophages in TB to induce MCP-1, MMP-1 and MMP-9 expression

Given the fact that MMP-1 can activate PAR-1,33 we asked whether this pathway might operate in TB. Alveolar macrophages are the first line of defense in the lungs and the initiators of peripheral monocyte recruitment into the lung’s inflammatory foci.28–31 Alveolar macrophages express CD14 and CD68 markers.34–36 IHC analysis of PAR-1 expression revealed that human alveolar macrophages in normal lungs constitutively express PAR-1. Of note, there were some macrophages in the interstitium of normal tissues (the tissue and space around the air sacs of the lung); we reasoned that this might be a displacement artifact produced while processing the samples (Figure 4, panel 1). Few macrophages in lung tissues from individuals with severe TB disease could be observed, which could be interpreted as macrophages (red for CD68) expressing PAR-1 (blue). Images taken at lower magnification of tissues presented in Figure 1, panel 2, corroborate this statement (Supplementary Figure S1).

PAR-1 was also expressed by epithelial cells in normal and disease lungs (Figure 4). Low PAR-1 expression in diseased tissues might result from the enzymatic activity of several proteases in the lung milieu of these severe cases that may digest several proteins, including PAR-1. In conjunction with this, PAR-1 may have been downregulated in response to activation by thrombin or/and MMP-1. Of note, the same anti-PAR-1 antibody was used to stain normal lung tissue, and both experiments were done simultaneously. Thus, lack of specificity of the antibody does not explain our results.

MMP-1 may activate PAR-1 in THP-1 cell exposed to M. tuberculosis sonicate

To test that whether MMP-1 activates PAR-1 in THP-1 cells exposed to sonicated M. tuberculosis H37Rv, we took advantage of...
the fact that PAR-1 is a G-coupled protein receptor and that, as such, PAR-1 undergoes recycling upon activation by thrombin or MMP-1. We focused our analysis of PAR-1 expression in THP-1 cells expressing a phenotype close to that of macrophages. We observed by fluorescence-activated cell sorting analysis that a low proportion of quiescent THP-1 cells expressed CD14 and that a very low proportion of these cells expressed CD16 (Figure 5, panels 1 and 2, section a). CD14-positive/CD16-negative THP-1 cells also expressed CD68 (data not shown). Exposure of THP-1 cells to sonicated H37Rv M. tuberculosis mainly induced their differentiation into CD14-positive/CD16-negative cells (Figure 5, panels 1 and 2, section b). Interestingly, none of the compounds or peptides used perturbed the differentiation of THP-1 cells into CD14-positive/CD16-negative cells (Figure 5, panels 1 and 2, section c). We next gated into CD14-positive/CD16-negative THP-1 cells to determine whether PAR-1 expression is regulated by exposure of these cells to sonicated H37Rv M. tuberculosis ± MMP-1 inhibitor 4-Aminobenzoyl-Gly-Pro-D-Leu-D-Ala hydroxamic peptide.
We then asked whether the CCR2 inhibitor RS504393 would provide similar results because MCP-1 activates CCR2 and through this it potentiates the expression of MMP-1 by M. tuberculosis-stimulated THP-1 cells (Figure 3, panel 1). Approximately 50% of the gated CD14-positive/CD16-negative quiescent THP-1 cells expressed PAR-1 (Figure 5, panel 2, section d). Once again, exposure of THP-1 cells to sonicated H37Rv M. tuberculosis significantly downregulated (to 3.39%) the expression of PAR-1 (d versus e: OR = 28.6, 95% CI = 20.2–41.1, P < 0.00001; Figure 5). The addition of CCR2 inhibitor to M. tuberculosis-stimulated THP-1 cells preserved the expression of PAR-1 in approximately 35% of the cells (f versus e: OR = 15.24, 95% CI = 11.1–21.5; P < 0.00001; Figure 5, panel 2). Notably, THP-1 cells cultured under any of the aforementioned conditions did not express thrombin (data not shown). Hence, we considered that this results added evidence that MMP-1 could be activating PAR-1 in this in vitro system and inducing recycling of this receptor. Because we partially recovered PAR-1 cell membrane expression by treating M. tuberculosis-antigen-activated THP-1 cells with a MMP-1 or a CCR2 inhibitor, we do not exclude the possibility that other macrophage proteases might be acting on PAR-1 as well.

Treatment of H37Rv M. tuberculosis-infected THP-1 cells with PAR-1 inhibitor SCH79797 downregulates MMP-1-induced MCP-1, MMP-1 and MMP-9.

We first used the SCH79797 PAR-1 inhibitor to test whether PAR-1 inhibition regulates the expression of MCP-1, MMP-1, MMP-9 and the TIMPs by M. tuberculosis-stimulated THP-1 cells. We observed that PAR-1 inhibitor SCH79797, at nanomolar concentrations (50 nM), potently downregulated MCP-1, MMP-1 and MMP-9 mRNA expression by THP-1 cells stimulated with sonicated H37Rv M. tuberculosis (Figure 6, panel 1). This inhibitor also significantly downregulated the expression of the TIMPs (Figure 6, panel 1). To confirm that MMP-1 activates PAR-1, we added exogenous human purified MMP-1 to M. tuberculosis-stimulated THP-1 cells and observed that exogenous MMP-1 increased MCP-1, MMP-1 and MMP-9 expression to levels that were higher than those observed in cultures of THP-1 cells stimulated with sonicated H37Rv M. tuberculosis alone (Figure 6, panel 2). Addition of PAR-1 inhibitor to THP-1 cells stimulated with sonicated H37Rv M. tuberculosis in the presence of human purified MMP-1 significantly decreased the expression of MCP-1, MMP-1 and MMP-9 (Figure 6, panel 1). Thus, we confirmed that MMP-1 activation of PAR-1 regulates the expression of MCP-1, MMP-1 and MMP-9 by M. tuberculosis-stimulated THP-1 cells.

Based on the results described above, we concluded that PAR-1 activation by MMP-1 emerged as a key event in M. tuberculosis-induced and MCP-1-potentiated inflammatory response in vitro. Thus, we further tested the effect of SCH79797 PAR-1 inhibitor on the expression of MCP-1, MMP-1, MMP-9 and additional 31 000 annotated genes in M. tuberculosis H37Rv-infected THP-1 cells, using an entire genome expression microarray.

We first selected those genes regulated by H37Rv M. tuberculosis infection. To do so, we compared the gene-expression profiles of infected cells and noninfected cells and selected those genes significantly regulated, according to our stringent parameters (Table 6). We then followed the effect of treatment on the expression of these genes by comparison of infected cells treated with SCH79797 (50 nM) versus infected cells untreated. Among those genes significantly regulated by PAR-1 inhibitor treatment were our primary target genes MCP-1 (CCL2), MMP-1 and MMP-9 (Table 6). In addition, IL-7 receptor (IL7R), toll-like receptor 8 (TLR8) and SPP-1 genes were also modulated by this treatment (Table 6). Thus, we confirmed the downregulation of MCP-1, MMP-1 and MMP-9 by PAR-1 inhibitor in M. tuberculosis-infected cells. Interestingly, PAR-1 treatment did not significantly modify the expression TNFα in response to infection (Table 6), which is required to mount a protective immune response against M. tuberculosis.37 In addition, CD14 gene expression was upregulated by M. tuberculosis infection, in concordance with the notion that infection causes maturation of THP-1 (Table 6).38 PAR-1 treatment of THP-1 cells did not modify significantly this trend (Table 6). We further confirmed the downregulation of MCP-1, MMP-1 and MMP-9 by PAR-1 inhibitor treating the concentrations of functional proteins in supernatants of THP-1 cells infected with M. tuberculosis H37Rv (Table 7).

We also analyzed those genes that were not regulated by infection per se, but were significantly regulated by PAR-1 treatment in infected cells. Of note, CCR2 (the receptor of MCP-1) and TIMP-3 were differentially and significantly downregulated by PAR-1 treatment of those cells (Supplementary Table S2). We did not observe significant differences in the colony forming unit in infected THP-1 cells treated with PAR-1 inhibitor or untreated (Table 7). In our model, PAR-1 has a significant role in the amplification of the inflammatory response to M. tuberculosis infection (Supplementary Figure S2).
DISCUSSION

Our studies in human populations combined with our *ex vivo* analysis of tissues from TB cases, and our *in vitro* model using the human THP-1 monocytic cell line, led us to conclude that the −2518 MCP-1 genotype GG and the −1607 MMP-1 genotype 2G/2G are key genetic loci associated with the expression of severe TB disease. The presence of both genotypes increases by 13-fold the chance of developing severe TB disease, by 3.864-fold the chance of a delayed sputum conversion from positive to negative and by 6.5-fold the chance of developing extensive areas of lung fibrosis. Moreover, the two-locus genotype −2518 MCP-1 GG and −1607 MMP-1 2G/2G operates with high penetrance in this population because 83% of the carriers expressed severe disease. Neither the −2518 MCP-1 genotype GG nor the −1607 MMP-1 genotype

Figure 4. PAR-1 is expressed in alveolar macrophages from normal lungs, whereas only few macrophages expressed PAR-1 in diseased lung tissues from severe TB cases. We used double IHC analysis of PAR-1 and CD68 in paraffin-embedded normal (panel 1) and diseased lung (panel 2). Sections b, c and d show three different normal persons (panel 1). One representative image from three of the six TB cases that underwent surgery to remove damaged tissue is shown in panel 2. In each panel, section a shows the negative control (irrelevant antibodies isotype control). Sections b, c and d show PAR-1 (blue) and CD68 (dark red) macrophages. Images were acquired at ×200 total magnification.
Figure 5. Downregulation of PAR-1 expression by exposure of THP-1 cells to sonicated H37Rv M. tuberculosis and counter effect of MMP-1 and CCR2 inhibitor. We used three-color fluorescence-activated cell sorting analysis for these experiments. Exposure of quiescent THP-1 cells to sonicated H37Rv M. tuberculosis for 24h induced the differentiation of these cells into CD14-positive/CD16-negative cells. In panels 1 and 2, section a shows a low proportion of CD14-positive/CD16-negative cells in quiescent THP-1 cells, section b shows an increment in the proportion of CD14-positive/CD16-negative THP-1 cells in response to sonicated H37RV M. tuberculosis exposure and section c shows minimal (nonsignificant) variation in this response to sonicated H37Rv M. tuberculosis exposure in the presence of the MMP-1 inhibitor (Panel 1) or CCR2 inhibitor (panel 2). In sections d, e and f of panel 1, we show that the presence of MMP-1 inhibitor 4-Aminobenzoyl-Gly-Pro-D-Leu-D-Ala hydroxamic peptide prevents the downregulation of PAR-1 expression by THP-1 cells exposed to sonicated H37Rv M. tuberculosis exposure. In sections d, e and f of panel 2, we show that the presence of CCR2 inhibitor RS504393 also prevents the downregulation of PAR-1 expression by THP-1 cells exposed to sonicated H37Rv M. tuberculosis exposure. We acquired 100,000 events for experiments in panel 1, whereas we acquired 10,000 events for experiments in panel 2, according to the number of live cells gathered in each experiment. Of note, the MMP-1 inhibitor was dissolved in incomplete RPMI, whereas the CCR2 inhibitor was dissolved in DMSO to obtain a DMSO final culture concentration of 0.01%. Three experiments were done for the assessment of each inhibitor.
2G/2G alone were sufficient to produce a severe disease. Interestingly, a higher proportion of TB patients carrying the susceptible two-locus genotype — 2518 MCP-1 GG and – 1607 MMP-1 2G/2G has had BMI values at first visit lesser than the median BMI value of the entire sample. Interestingly, weight loss is per se a key marker of severity of TB disease in animal models. Altogether, our observations have important implications when the interest is to detect, as early as possible, those persons who are at increased chance of developing a severe TB disease, may remain contagious for a longer period than any other TB case and will develop a poor...
health-related quality of life. We must remember that these TB cases were tightly followed up, which may have decreased the chance of abandonment of treatment and progression to a potentially lethal illness. This may also have implications for the treatment time required to attain spurtum smear test conversion in TB cases infected with multidrug-resistant M. tuberculosis strains and the increased mortality observed among them.

Six TB cases in carriers of the two-locus genotype – 2518 MCP-1 GG and 1607 MMP-1 2G/2G expressing severe disease (score > 44) underwent surgery to remove damaged lung tissue versus none in the groups of carriers of any other combination of genotypes at these two loci. The IHC analysis of these lung tissues revealed macrophages expressing copious amounts of MCP-1/ CCL2, MMP-1 and MMP-9. Cells of other lineages (CD68-negative) were also present in the lesions and express these factors, indicating a possible cross-talk between macrophages and, perhaps, lung epithelial cells. Our IHC analysis is in agreement with results from a genome-wide comparison of gene expression in human granulomatous lesions. Access to their database allowed us to rank the genes according to the magnitude of the P-values obtained from Student’s t-tests. Of note, levels of specific mRNAs from nonstimulated cells were not significantly different from those obtained from cultures that proceeded with the PAR-1 inhibitor alone. Panel 2: the effect of 1 nM exogenous human purified MMP-1 on THP-1 cells exposed to 5 μg ml⁻¹ sonicated H37Rv M. tuberculosis was assessed. We also assessed the effect of 1 nM exogenous human purified MMP-1 on THP-1 cells exposed to 5 μg ml⁻¹ sonicated H37Rv M. tuberculosis in the presence or absence of 50 nM concentration of PAR-1 SCH97977-inhibiting compound. We measured the relative changes in MCP-1, MMP-1 and MMP-9 gene expression by real-time PCR as explained above. Cultures proceeded in 500 μl of incomplete RPMI. The results presented are from three independent experiments showing the mean and s.d. We consistently observed significant differences in the mean values across variables (Kruskal–Wallis -tests). We tested serum and plasma samples and observed the highest serum levels of MCP-1 and the highest plasma levels of MMP-1 and MMP-9 in carriers of the two-locus genotype – 2518 MCP-1 GG and 1607 MMP-1 2G/2G as compared with levels of these molecules in carriers of any other relevant combination of genotypes at those loci. It was unexpected to see the highest levels of MMP-9 in carriers of the two-locus genotype – 2518 MCP-1 GG and 1607 MMP-1 2G/2G. We thought that preceding molecular events involving increased production of MCP-1 and MMP-1 levels might explain why these TB cases expressed the highest levels of MMP-9. We first tested whether endogenous MCP-1 and MMP-1 were involved in the induction of MMP-9 using CCR2-inhibiting compound RS504393 and MMP-1-inhibiting 4-Aminobenzoyl-Gly-Pro-D-Leu-D-Ala hydroxamic peptide. We clearly established in vitro that the MCP-1/MMP-1 loop drives the expression of MMP-9 to its highest levels in THP-1 cells activated by sonicated H37Rv M. tuberculosis. Moreover, addition of exogenous human MMP-1 to THP-1 cells activated by sonicated H37Rv M. tuberculosis potentiated significantly the production of MMP-9. Hence, these in vitro data may explain why we observed the highest levels of MMP-9 in carriers of the two-locus genotype – 2518 MCP-1 GG and 1607 MMP-1 2G/2G. Given these observations, we concluded that it is very likely that the MCP-1/MMP-1/MMP-9 pathway is driving an intense inflammatory response to infection in lung tissues of patients carrying that susceptible two-locus genotype. Notably, MMP-9 not only helps in recruiting macrophages to the inflammatory foc27 but also degrades collagen by-products of MMP-1-digested fibrilar collagen.41 Because it is known that MMP-1 cleaves the PAR-1N-terminal exodomain to generate a SFLLRN-containing motif that activates the PAR-1 signaling pathway,33 we investigated whether this pathway may operate in TB. We observed by IHC that alveolar macrophages in normal lungs constitutively express PAR-1, whereas in few macrophages express PAR-1 in TB cases suffering from severe lung disease. Nevertheless, we concluded that lung macrophages express PAR-1 in healthy and to a lesser extent in disease lung parenchyma. Our fluorescence-activated cell sorting analysis experiments suggested that MMP-1 activates PAR-1 in monocytes cells exposed to M. tuberculosis antigens. We first established that as a result of exposure to M. tuberculosis antigens, PAR-1 expressed in CD14-positive/CD16-negative THP-1 cells was significantly downregulated, which may indicate that an specific ligand was activating, and subsequently inducing the recycle of this receptor. Addition of MMP-1 inhibitor 4-Aminobenzoyl-Gly-Pro-D-Leu-D-Ala hydroxamic peptide to the cultures significantly neutralized M. tuberculosis antigen-induced downregulation of PAR-1 expression in these cells, indicating that MMP-1 was one of the molecules activating PAR-1. Furthermore, in agreement with our observation that MCP-1 potentiates MMP-1 secretion by THP-1 cells exposed to sonicated H37Rv M. tuberculosis, we also observed that the addition of CCR2 inhibitor RS504393 prevented the activation and consequently the downregulation of PAR-1 expression by CD14-positive/CD16-negative THP-1 cells. Because THP-1 cells did not express thrombin under those conditions, we concluded that MMP-1 might be one of the proteases activating PAR-1, and thereby inducing the recycling of activated PAR-1 in our in vitro system.

We also conducted studies to directly determine the possible role of PAR-1 activation by MMP-1 in TB, adding exogenous human MMP-1 to THP-1 cells activated with sonicated M. tuberculosis H37Rv. These experiments corroborated a link between the MCP-1/MMP-1 upregulation of MMP-9 with activation of PAR-1 in monocytes/macrophages. Addition of PAR-1 inhibitor SCH97977 at nanomolar concentration to THP-1 cells stimulated with sonicated M. tuberculosis H37Rv or THP-1 cells infected in vitro with M. tuberculosis H37Rv significantly downregulated MCP-1, MMP-1 and MMP-9 expression, whereas the TIMPs remained poorly expressed. Likewise, addition of this PAR-1 inhibitor, at nanomolar concentration, to cultures of M. tuberculosis-stimulated THP-1 cells that proceeded in the presence of exogenous human purified MMP-1 resulted in inhibition of MCP-1, MMP-1 and MMP-9 expression. Interestingly, this PAR-1 inhibitor downregulated the expression of CCR2 in infected cells, which may contribute to decrease the effect of excessive MCP-1 production.

Figure 6. PAR-1 inhibitor SCH97977 regulates the expression of MCP-1, MMP-1 and MMP-9 in THP-1 monocytes cells stimulated by sonicated H37Rv M. tuberculosis and THP-1 cells stimulated by sonicated H37Rv M. tuberculosis in the presence of exogenous purified human MMP-1. Panel 1: we measured the relative changes in MCP-1, MMP-1 and MMP-9 and TIMP gene expression by real-time PCR. Data are presented as the fold change in gene expression normalized to the endogenous reference gene PDHB and relative to untreated controls. The effect of 50 nM concentration of SCH97977 PAR-1-inhibiting compound was assessed following 24 h in vitro stimulation of THP-1 cells with 5 μg ml⁻¹ sonicated H37Rv M. tuberculosis. Cultures proceeded in 500 μl incomplete RPMI. PAR-1 SCH97977-inhibiting compound was diluted with DMSO and dispensed in 5 μl volume to produce a final culture concentration of 0.01% DMSO. We also added 5 μl of DMSO to control cultures. The results presented are from six independent experiments showing the mean and s.d. We consistently observed significant differences in the mean values across variables (Kruskal–Wallis -tests). Of note, levels of specific mRNAs from nonstimulated cells were not significantly different from those obtained from cultures that proceeded with the PAR-1 inhibitor alone. Panel 2: the effect of 1 nM exogenous human purified MMP-1 on THP-1 cells exposed to 5 μg ml⁻¹ sonicated H37Rv M. tuberculosis was assessed. We also assessed the effect of 1 nM exogenous human purified MMP-1 on THP-1 cells exposed to 5 μg ml⁻¹ sonicated H37Rv M. tuberculosis in the presence or absence of 50 nM concentration of PAR-1 SCH97977-inhibiting compound. We measured the relative changes in MCP-1, MMP-1 and MMP-9 gene expression by real-time PCR as explained above. Cultures proceeded in 500 μl of incomplete RPMI. The results presented are from three independent experiments showing the mean and s.d. We consistently observed significant differences in the mean values across variables (Kruskal–Wallis -tests) when testing MCP-1, MMP-1 and MMP-9. We show corrected P-values obtained from Student’s t-tests. The inhibitor’s concentration was selected from dose–response experiments.
| Target ID | CYTOBAND | ProbeID | NINT AVG. Sig. | NINT ARRAY SD | YINT AVG. Sig. | YINT ARRAY SD | Diff. Score | YINT Diff. P-val | Log-ratios [YINT/NINT] | YINT ARRAY SD | YINT Diff. Score | YINT Diff. P-val | Log-ratios [YINT/NINT] |
|-----------|----------|---------|----------------|---------------|----------------|---------------|------------|-----------------|----------------------|---------------|----------------|-----------------|------------------|
| 1041251   | 6q23.1   | 3425003 | 1.56           | 0.0035        | 0.99998        | 0.0035        | 0.99998    | 0.0035          | 0.99998              | 0.0035        | 0.99998        | 0.0035          | 0.99998          |

Abbreviations: NINT, not infected not treated; YINT, yes infected yes treated. Data from those genes with scanning detection $p$-values $< 0.05$, differentially express $p$-values $< 0.05$ after false discovery rate correction (FDR, $p$-value), and Log ratios $> 1.5$ (regardless of the orientation) are shown. The differential scores (Diff. score) are a function of the $P$-values (Illumina Inc.). Log ratios are the logarithm in base 2 of the average signal (AVG. sig.) ratios. We also show the array $s.d.$ of the average signals (Array. SD). We show highlighted in red the results from our primary targets. In black bold, we show genes with scanning detection $p$-values $< 0.05$, differentially express $p$-values $< 0.05$ before false discovery rate correction and Log ratios $> 1.5$ (regardless of the orientation). All cultures proceed and were processed according to the protocols described in Materials and methods, including washing steps.

The similarities between the results from the reanalysis of data from a genome-wide comparison of gene expression in human granulomatous lesions mentioned above, and the data we have obtained from *M. tuberculosis* infections of THP-1 cells, provide physiological relevance to our in vitro observations. It is interesting to study the effects of the compound or peptide inhibitors we have tested, alone or in combinations, on primary human CD14 + CD16 – and CD14 + CD16 + macrophages. Likewise, because we did not attain an efficient transformation of a significant proportion of THP-1 cells with
upregulation in response to infection is not modified in THP-1 cells by treatment with SCH97979. PAR-1 inhibitor is encouraging because this is a factor critically involved in the development of cellular immune response against *M. tuberculosis* infection. Studies in animal models are needed to confirm in vivo these molecular mechanisms and to confirm that the PAR-1 inhibitor SCH97979 constitutes a promising candidate for the treatment of MCP-1/MMP-1-mediated tissue damage in TB. Thus, our finding that MMP-1, through PAR-1 activation, is driving a hyper-inflammatory response to infection highlights key molecular events for the development of new clinical interventions to attenuate tissue damage.

**MATERIALS AND METHODS**

**Ethics statement**

All subjects provided informed consent, under protocols approved by the Institutional Review Boards of The Methodist Hospital Research Institute in Houston (TX, USA) and the Peruvian Ministry of Health.

**Recruitment base**

People from Peru are of Hispanic ancestry whose genetic composition consists mainly of an admixture of Amerindians and Spaniards. People with this genetic composition are the main ethnic group in most Latin-American countries and of Hispanics living in the United States. Peru is a moderate-to-high tuberculosis-burdened country (http://www.who.int). The TB incidence in 2010 was 118 per 100,000 person-years and the prevalence of TB was 187 per 100,000 person-years (http://www.who.int). The existence of government-run, community-based programs designed to detect new TB cases in Peru facilitates identification of well-characterized study subjects (http://www.minsa.gob.pe). We recruited our cases using a sentinel surveillance network comprised of primary (community) health centers under the jurisdiction of two National Hospitals (serving a total population of 10 million individuals) in Lima Province, the Hospital Hipolito Unanue and the Hospital Maria Auxiliadora. All TB cases were residents of communities sharing similar TB incidence, socioeconomic status and life styles, and very likely similar *M. tuberculosis* strains. Most of the individuals suffering from TB in Peru are of low socioeconomic status (http://www.who.int and http://www.minsa.gob.pe).

**Inclusion and exclusion criteria**

We conducted a case–case study of homogeneous populations of Peruvian Mestizo ancestry. From July 2009 to July 2011, we recruited 224 TB cases meeting our inclusion/exclusion criteria. TB cases were unrelated to the third generation as determined by a questionnaire, aged >18 to ≤50 years old. They were of Peruvian ethnicity to the third generation, increasing the likelihood that this population is ethnically homogeneous. Patients were not HIV-seropositive and/or did not have comorbidities affecting immunity, including autoimmunity, cancer, organ transplantation and primary immunodeficiency, were not on therapy with immunosuppressive drugs (such as corticosteroids) and did not suffer from endocrine disorders, or chronic cardiopulmonary, hepatic or renal disease. Patients did not suffer from alcoholism and were not illegal drug users. BMI was used as an indicator of malnourishment in adults.44,45 The BMI was determined based on weight and height measured by a nurse, as we did before.1,2 We selected TB patients meeting the following criteria: (1) Completeness of demographic and clinical data; (2) All TB cases were new with a disease of not more than 3 months of duration. We excluded cases of recurrent or reinfeeted TB. Recruitment of new cases avoids confounding of data by unmeasured (unknown) factors that increase the chance of recurrent or reinfeeted TB; (3) Patients who developed TB disease within a period of 2 years following the most recent close exposure to an index case; (4) TB patients identified at the local (community) TB control center to avoid selection bias of choosing a disproportionate number of TB cases with a severe disease presentation that needed specialized clinical interventions at secondary or tertiary hospitals. Physicians at the local TB control center identified all cases; and (5) Patients with positive adherence to treatment. Patients had: (i) signs and symptoms of active TB; (ii) a full-size posteroanterior chest X-ray suggestive of active pulmonary TB; (iii) disease confirmed by at least one of three sputum smear test; and (iv) monthly sputum smear tests to

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**Table 7. PAR-1 inhibitor treatment of infected THP-1 cells downregulates MCP-1, MMP-1 and MMP-9 protein secretion, but does not modify *Mycobacterium tuberculosis* intracellular proliferation**

| Culture condition/measurement | M. tuberculosis-infected, untreated | M. tuberculosis-infected, treated | Two-sample t-test |
|-----------------------------|-----------------------------------|---------------------------------|-----------------|
| MCP-1 (pg ml⁻¹ ± s.d.)       | 136 ± 39.7                         | 0                               | *P* = 0.004     |
| MMP-1 (pg ml⁻¹ ± s.d.)       | 744.4 ± 103.4                      | 0                               | *P* < 0.00001  |
| MMP-9 (pg ml⁻¹ ± s.d.)       | 951 ± 23                           | 0                               | *P* < 0.0001   |
| CFU per ml (10⁴)             | 9 ± 2.65                           | 8.3 ± 3.1                       | n.s.            |

**Abbreviations:** CFU, colony forming unit; n.s., not significant. Soluble factors were determined in supernatants using enzyme-linked immunosorbent assay test for MCP-1 (BD Biosciences) and functional specific-peptide-cleave enzyme-linked immunosorbent assay tests for MMP-1 and MMP-9 (R&D Systems). Cell pellets lysed in Triton X-100 were used to recover intracellular bacteria and processed as indicated in material and methods to obtain the CFU counts. The values for uninfected untreated negative control THP-1 cells were zero for MCP-1, MMP-1 and MMP-9.
follow-up sputum smear test conversion from positive to negative. New TB patients in Peru received a four-drug standardized treatment consisting of 300 mg of rifampicin + 300 mg of isoniazid + 1500 mg of pyrazinamide + 1200 mg of ethambutol daily for 2 months, followed by 300 mg of rifampicin + 300 mg of isoniazid twice a week for 2 months http://www.minsa.gob.pe.

### The severity score

We defined disease duration as the length of time since the first symptom was noticed by the patient to the time of sampling. The total score is the sum of a score based on the assessment of clinical signs and symptoms plus a score based on the assessment of posteroanterior full-size chest X-rays (total from the independent assessment of the two tables (2)). BML in adults was assessed according to the World Health Organization classification.

### Blood samples and SNP analysis

Genomic DNA was isolated from blood cell pellets using DNA extraction kits (Qiagen, Valencia, CA, USA). We genotyped the −2518A > G SNP in MCP-1 (rs1024611), the −1607_1608insG variant in MMP-1 (rs1799750) and 42 genomic control SNPs. The rs1024611 and rs1799750 SNPs were genotyped in duplicate using the tetra-primer ARMS PCR technique. We frequent in this population, we used them as reference alleles.

### Culture conditions and real-time PCR assay

For the assessment of MCP-1, MMP-1, MMP-9, Timp-1, -2, -3 and -4 expression, we used the 7500 Fast Real-Time PCR System and assay-on-demand primers for MCP-1 (ID Hs00234140), MMP-1 (ID Hs0023958), MMP-9 (ID Hs00234579), Timp-1 (ID Hs00171558), Timp-2 (ID Hs00109131), Timp-3 (ID Hs00227214), and the endogenous reference gene PDHB (pyruvate dehydrogenase beta). ID Hs00166850 (Applied Biosystems, San Ramon, CA, USA). To calculate the relative quantity of mRNA expression, we used the 2−ΔΔCT method implemented in the software (Applied Biosystems).

### Three-color fluorescence-activated cell sorting analysis

THP-1 cells cultured as described above were harvested and washed twice in cold incomplete RPMI, then resuspended in 100 μL of cold incomplete RPMI containing 10 μg/mL of human IgG (Sigma) and incubated on ice for 15 min. Cells were washed once in phosphate-buffered saline + 10% bovine serum albumin (Sigma) and resuspended at 5 × 10^6 cells per 100 μL of this buffer. FITC-anti-human CD14 mouse monoclonal antibody 61D3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Per CP-Cy5.5 anti-human CD16 mouse monoclonal antibody 3A5 (BD Pharmingen, San Diego, CA, USA), and human CD14 mouse monoclonal antibody DJ130c (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), were used in combination with p-aminophenylmercuric acetate (EMD Chemicals, Billerica, MA, USA) or irrelevant normal mouse IgG antibody (R&D Systems). We used 1 : 4 dilution of FITC-anti-human CD14 mouse monoclonal antibody 61D3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and the endogenous reference gene PDHB (pyruvate dehydrogenase beta). ID Hs00166850 (Applied Biosystems, San Ramon, CA, USA) was used for monitoring the expression levels of the housekeeping gene PDHB and MCP-1, MMP-1, MMP-9 and TIMPs.

### Infections of THP-1 cells and gene expression microarrays

We used the M. tuberculosis H37Rv (ATCC) grown in Middlebrook 7H9 broth supplemented with ADC enrichment (Difco Laboratories, Detroit, MI, USA), 2% glucose and 1% glycerol. Cultures proceeded at 37°C and constant shaking to mid-log growth phase (7–10 days). The bacteria were quantified using McFarland equivalence turbidity standards (Thermo Scientific). Fresh cultures of 10^6–10^8 bacilli per mL bacteria were immediately used for infections. To determine M. tuberculosis colony forming unit cell pellets were obtained by centrifugation at 1500 g rpm and the cell pellets were lysed in 0.1% Triton X-100 (Sigma) before preparation.
of plating dilutions. We counted colony forming units per ml by serial titration of cultures 1:10, 1:100 and 1:1000 on 7H9 plated on 7H11 agar plates, and left in culture for 3 weeks at 37 °C.

THP-1 cells were infected with M. tuberculosis bacilli at a multiplicity of infection of five bacteria per cell in complete RPMI (10% fetal bovine serum) without antibiotics. We used this culture media throughout the experiment. controls. THP-1 cells and M. tuberculosis bacilli were incubated for 6 h at 37 °C in 5% CO2, then washed three times in incomplete RPMI and plated at 1 million cells per 0.5 ml of culture media in 48-well plates. We then added the PAR-1 inhibitor SCH779779 (Tocris) at a final culture concentration of 50 nM. The PAR-1 inhibitor was previously diluted in dimethyl sulfoxide at a 5-μM working concentration to dispense a 5 μl volume per well and to produce the desired concentration of PAR-1 inhibitor and a final culture concentration of 0.01% dimethyl sulfoxide. Control cultures of infected cells contained 0.01% dimethyl sulfoxide alone. Then the cultures proceeded for additional 18 h when we proceeded to harvest the THP-1 cells for isolation of RNA or for colony forming unit determinations and to harvest the culture supernatants for enzyme-linked immunosorbent assay tests. We did not induce differentiation of THP-1 cells previous to infection because exposure of THP-1 cells to M. tuberculosis (or M. tuberculosis sonicate) induces differentiation of these cells into macrophage-like adherent cells.

Total RNA from these samples was extracted using Trizol followed by total RNA purification using Qiagen Kit as per manufacturer’s instructions. We assessed the quality of RNA using Agilent RNA 6000 Nano Kit. Gene-expression profiling was performed using the Illumina HumanHT-12v4 BeadChip (Illumina Inc.). Two hundred nanograms of total RNA were labeled using a TotalPrep RNA Amplification Kit (Applied Biosystems) to obtain biotinylated-cRNA. We quantified and ascertainment the fragment size using the Agilent RNA 6000 Nano Kit. We loaded the beadchip’s arrays with 750 ng of biotinylated-cRNA, and left them to hybridize overnight. We then washed and scanned the BeadChip arrays according to the manufacturer’s instructions. The chips were scanned using the Illumina BeadArray Reader.

Statistical analysis

Statistical analysis was done with Intercooled STATA10 software (Stata Corporation, College Station, TX, USA). Chi-square or Student’s t-test was used to assess differences between categorical or continuous variables, respectively. The severity of disease was first established by expert clinicians in the field. Each TB case had a clinical diagnosis of disease severity as: non-severe or severe disease. Once the severity scores were obtained, we classified the cases according to whether they had severity score values > or ≤ the median. We used the Kappa statistic and a modified Fleiss and vine-Haenszel scale to determine the agreement between (i) an expert clinician assessment of the cases and (ii) the severity of disease classification obtained by another clinician using our clinical charts and Table 2 (severity scores). According to this scale, the interpretation of Kappa coefficients is as follows: <0.0 = less than chance agreement, 0.01–0.2 = slight agreement, 0.21–0.40 = fair agreement, 0.41–0.60 = moderate agreement, 0.61–0.80 = substantial agreement, and 0.81–1.0 = perfect agreement.43 We treated the severity scores as a dependent binary random variable (0 = non-severe score (score ≤ 44); 1 = severe disease (score > 44); Table 3) and assessed the contribution on severity of disease of each locus and the two-locus genotypes, adjusting for age and gender using logistic regression analysis. We performed Pearson’s goodness-of-fit χ2-tests to determine whether the observed models differed from the predicted. We used the non-parametric Kruskal–Wallis test, followed by the Bonferroni least significant difference test for multiple comparisons to analyze differences in the mean severity score stratified according to genotypes. Spearman’s test was used to evaluate correlations. We used multiple 2 × 2 tables, with genotype arranged in an ordinal scale, and χ2 and the McNemar–Haenszel statistics to test for genotypes associations with delayed (>3 months) sputum smear test conversion primarily to obtain valid estimates for association for each stratum and to assess dose effects and homogeneity of the ORs across strata. To test for homogeneity of OR, we used the Breslow and Day’s test provided in the Sata10 output. One-way ANOVA followed by a Bonferroni least significant difference test was used to evaluate correlation between the assessment of serum plasma levels of MCP-1, MMP-1 and MMP-9 and results from in vitro experiments designed to test contributions of the MCP-1/CCR2, MMP-1 and PAR-1 pathways in models of M. tuberculosis-induced expression of inflammatory factors by THP-1 cells.

We subtracted the background to the raw data obtained from the Illumina platform and proceeded to normalized data using quantile normalization.44 We excluded probes showing detection P-values < 0.05. All the statistical analyses of the expression data were conducted using the GenomeStudio package (Illumina Inc.). The P-values were obtained using false discovery rate for multiple testing adjustments.45 Given that the scores obtained for each gene were a function of the P-values, we calculated the log2 of the average signal’s ratios (log ratios) to assess the magnitude and orientation (up- or downregulation) of the gene regulation. We declare significant change in expression when false discovery rate-adjusted P-values were ≤ 0.05 and the log2 ratios > 1.5, regardless of the orientation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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