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High Matrix Metalloproteinase Production Correlates with Immune Activation and Leukocyte Migration in Leprosy Reactional Lesions▽

Rosane M. B. Teles,[1] Rose B. Teles,[1] Thais P. Amadeu,[1,3] Danielle F. Moura,[1] Leila Mendonça-Lima,[2] Helen Ferreira,[1] Ítalo M. C. F. Santos,[1] José A. C. Nery,[1] Euzenir N. Sarno,[1]* and Elizabeth P. Sampaio[1]†

Leprosy Laboratory,[1] and Laboratory for Functional Genomics and Bioinformatics,[2] Oswaldo Cruz Institute, FIOCRUZ, Rio de Janeiro, RJ, Brazil, and Department of Histology and Embryology, UERJ, Rio de Janeiro, RJ, Brazil[3]

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Gelatinases A and B (matrix metalloproteinase 2 [MMP-2] and MMP-9, respectively) can induce basal membrane breakdown and leukocyte migration, but their role in leprosy skin inflammation remains unclear. In this study, we analyzed clinical specimens from leprosy patients taken from stable, untreated skin lesions and during reactional episodes (reversal reaction [RR] and *erythema nodosum leprosum* [ENL]). The participation of MMPs in disease was suggested by (i) increased MMP mRNA expression levels in skin biopsy specimens correlating with the expression of gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α), (ii) the detection of the MMP protein and enzymatic activity within the inflammatory infiltrate, (iii) increased MMP levels in patient sera, and (iv) the *in vitro* induction of MMP-9 by *Mycobacterium leprae* and/or TNF-α. It was observed that IFN-γ, TNF-α, MMP-2, and MMP-9 mRNA levels were higher in tuberculoid than lepromatous lesions. In contrast, interleukin-10 and tissue inhibitor of MMP (TIMP-1) message were not differentially modulated. These data correlated with the detection of the MMP protein evidenced by immunohistochemistry and confocal microscopy. When RR and ENL lesions were analyzed, an increase in TNF-α, MMP-2, and MMP-9, but not TIMP-1, mRNA levels was observed together with stronger MMP activity (zymography/*in situ* zymography). Moreover, following *in vitro* stimulation of peripheral blood cells, *M. leprae* induced the expression of MMP-9 (mRNA and protein) in cultured cells. Overall, the present data demonstrate an enhanced MMP/TIMP-1 ratio in the inflammatory states of leprosy and point to potential mechanisms for tissue damage. These results pave the way toward the application of new therapeutic interventions for leprosy reactions.

Matrix metalloproteinases (MMPs) compose a family of zinc- and calcium-dependent proteolytic enzymes responsible for extracellular matrix (ECM) remodeling and the regulation of the *trans*-ECM migration of leukocytes, an important step in inflammatory processes as well as infectious diseases. MMPs are functionally classified according to their relative substrate specificities but have been shown to overlap. Collagenases (MMP-1, -8, and -13) degrade type I collagen, whereas gelatinases A and B (MMP-2 and -9, respectively) degrade denatured type I (gelatin) and type IV collagens, a major component of the basement membrane. Collagenases are produced by many cell types including lymphocytes, granulocytes, astrocytes, and activated macrophages (10, 18).

MMP secretion takes place under tight regulatory mechanisms including transcriptional controls in addition to their release as proenzymes, requiring activation by specific proteases and cytokines present in the milieu (2, 3, 16). Also, the postactivation of MMPs is controlled by metalloproteinase tissue inhibitors (tissue inhibitor of MMP [TIMP]), a family of specific inhibitors, that bind to MMPs in a stoichiometric ratio. Thus, the matrix-degrading capacity of MMPs depends on the balance between MMP levels and the availability of extracellular TIMPs (5, 8). Due to their capacity to degrade basement membrane components, the gelatinases MMP-2 and MMP-9 are key molecules in leukocyte recruitment to inflammatory sites, which are indispensable for the containment of infection (27). However, excessive MMP secretion has been related to tissue damage in many inflammatory disorders (9, 25, 27).

High levels of MMP-9 detected in sera of patients have been considered to be biomarkers of disease activity (7, 31). Similarly, in tuberculosis (TB) pleurisy, the MMP-9 present in pleural fluid was associated with the presence of granulomas in the tissue and with protein staining in the mononuclear cells of the infiltrate (37). Several studies reported the ability of cytokines to modulate MMP production. In this connection, tumor necrosis factor alpha (TNF-α) was shown to upregulate MMP-9 and TNF neutralization for the purpose of abolishing MMP secretion (33). Gamma interferon (IFN-γ) could contribute to the activation of macrophages coexpressing TNF-α, MMP-2, and MMP-9 (6). Nevertheless, IFN-γ has been described to be mainly inhibitory (14, 17). In addition, MMP-9 secretion seems to be a common feature of mycobacterial infection since the induction of these enzymes in response to *Mycobacterium avium*, *Mycobacterium tuberculosis*, and *Mycobacterium bovis* BCG was reported (12, 28). Moreover, the addition of IFN-γ to BCG-infected murine macrophages inhibited MMP-9 secretion *in vitro* (28).

* Corresponding author. Mailing address: Leprosy Laboratory, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Av. Brasil 4365, Manguinhos, 21040-900 Rio de Janeiro, RJ, Brazil. Phone and fax: 00 55 21 2270-9997. E-mail: euzenir@fiocruz.br.

† Present address: Laboratory of Clinical Infectious Diseases, NIAID, NIH, Bethesda, MD 20892.

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Leprosy, an infectious disease caused by *Mycobacterium leprae*, is characterized by clinical patterns related to the immunological status of the patient. At the tuberculoid pole (tuberculoid leprosy [TT] and borderline tuberculoid [BT] forms), skin lesions with epithelioid granulomas are associated with a TH1 profile and bacterium-killing capacity (paucibacillary [PB] patients), whereas in lepromatous lesions (lepromatous leprosy [LL] and borderline lepromatous leprosy [BL]), diffuse macrophage infiltrates loaded with bacteria (multibacillary [MB] patients) are associated with the absence of a specific cellular immune response to *M. leprae*. During the course of the disease, new skin lesions may occur abruptly, characterized by dense macrophage and lymphocyte infiltration organized or not into granulomas typical of a reversal reaction (RR). In the lepromatous forms, however, the reactional state, referred to as *erythema nodosum leprosum* (ENL), has an abundance of neutrophils and is associated with acute systemic symptoms. The reactivation of the immune response and the upregulation of proinflammatory cytokines have been broadly documented for both forms of reaction (19–21, 34), and both are implicated in leprosy morbidity. Immunosuppressors such as corticosteroids and thalidomide have been the only treatment options available.

It was recently reported that MMPs may play a key role in promoting inflammatory skin damage. These enzymes can be produced by skin cells such as keratinocytes, Langerhans cells, and dermal fibroblasts (29, 36). However, no data on the role of MMPs in leprosy skin lesions currently exist. In order to investigate the participation of MMPs in the pathogenesis of leprosy, we analyzed skin and serum samples obtained from patients at different clinical moments. In addition, the role played by *M. leprae* in the induction of MMP production by blood cells *in vitro* was investigated. Our data support the hypothesis that MMPs may be implicated in the local and systemic responses to *M. leprae* infection, which may open new opportunities for therapeutic interventions in leprosy as well as leprosy reactions.

MATERIALS AND METHODS

Patients. Leprosy patients treated at the Leprosy Out-Patient Unit, Oswaldo Cruz Foundation, Rio de Janeiro, RJ, Brazil, were diagnosed according to the Ridley-Jopling classification (30). A total of 32 patients (24 males and 8 females; mean age ± standard deviation [SD] = 34.3 ± 14 years) were included in the study. Patients were classified as having LL ($n = 6$), BL leprosy ($n = 16$), borderline lepromatous-borderline tuberculoid (BB) leprosy ($n = 3$), or BT (T-lep) leprosy ($n = 7$). All patients were treated with multidrug therapy (MDT) (rifampin, dapsone, and clofazimine) as recommend by the World Health Organization. Eighteen out of the 32 patients (4 LL, 11 BL, and 3 BB [L-lep]) who presented with acute inflammatory reactional episodes (10 ENL and 8 RR patients) were also evaluated. The study was approved by the Institutional Ethics Committee of the Oswaldo Cruz Foundation. After written consent, biopsy specimens and blood were obtained from the patients and processed as described below. Biopsy specimens of 7 patients were taken before and during reactions (4 ENL and 3 RR patients); 14 patients (2 LL, 5 BL, and 7 BT patients) were evaluated solely at the moment of leprosy diagnosis and before the initiation of MDT.

Skin biopsy specimens and immunostaining. Biopsy specimens of skin lesions were bisected and processed for diagnostic procedures, fixed in formalin, and stained with hematoxylin and eosin or Wade-Fite stain. Immunostaining for the detection of MMP-2 and MMP-9 on frozen cryostat sections was performed by use of the immunoperoxidase method. Sections were acetone fixed, and the endogenous peroxidase was blocked with 0.3% H_2O_2. Nonspecific binding was blocked with goat normal serum for 1 h at room temperature. The sections were incubated overnight with the primary antibodies (Ab) anti-MMP-9 and anti-MMP-2 (1:50 dilution; R&D Systems). After washing, the sections were incubated with biotinylated secondary Ab (Dako, Carpinteria, CA) for 1 h at room temperature. The sections were rinsed, and the avidin-biotin complex (ABC; Vector Laboratories) was applied for an additional hour, followed by incubation in ABC reagent and the subsequent addition of substrate (3-amino-9-ethylcarbazole [AEC]; Vector Laboratories). Slides were counterstained with Mayer's hematoxylin and mounted. After omitting the secondary Ab, control sections were incubated with nonimmune serum and used as negative controls. The slides were examined under a Leica DMRB microscope (Leica Microsystems). To detect the possible colocalization of MMP-9 and CD68 (macrophage marker), double immunofluorescence was performed. Sections were incubated with monoclonal antibodies (MAbs) against human MMP-9 (1:100, IgG1; R&D Systems) and CD68 (1:100, IgG2a; Dako), followed by incubation with isotype-specific fluorochrome Alexa 488- or Alexa 568-labeled goat anti-mouse Ab (Invitrogen Life Technologies). No staining was detected after incubation with isotype-matched, irrelevant Ab. Images were recorded simultaneously via separate optical detectors and superimposed for colocalization analysis. The slides were then examined with a confocal microscope (TCS-SP MP inverted single-confocal laser scanning and two-photon laser microscope; Leica, Heidelberg, Germany). The percentage of colocalization between MMP and CD68 staining was calculated by use of the Andor IQ RGB analysis tool (Andor Technology, Ireland).

PBMC culture. Peripheral blood mononuclear cells (PBMC) were isolated from the heparinized venous blood of 7 leprosy patients by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density centrifugation. Cells were washed in phosphate-buffered saline (PBS) and suspended in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10% fetal calf serum (FCS; Gibco BRL, Gaithersburg, MD). Viability was estimated by trypan blue dye exclusion. A total of 2×10^6 PBMC were cultured in complete RPMI medium in 24-well plates at 37°C in a humidified CO_2 incubator and stimulated with *M. leprae* (10 μg/ml) or medium alone (control). After different time periods, culture supernatants were collected and frozen for quantification by enzyme-linked immunosorbent assay (ELISA), and the cells were processed for RNA isolation. For the zymography experiments, PBMC (10^6 cells) were cultured in complete medium with 1% FCS, stimulated or not with *M. leprae.* Afterwards, the supernatants were harvested after 24 h and kept frozen for posterior analysis. For the collection of serum samples, blood was allowed to clot, and serum aliquots were stored at −70°C until further use. Blood for cell isolation and serum samples from 4 healthy donors (blood bank) were also obtained and assayed in parallel.

RNA extraction and cDNA synthesis. Total RNA from biopsy specimens and cultured cells was extracted by using Trizol (Invitrogen) according to the manufacturer's instructions. In the case of biopsy specimens, RNA from the dermis was processed separately (42), and tissue was homogenized with a Politron PT-3000 apparatus (Brinkman, Westbury, NY) in 1 ml Trizol. From all samples, 1 μg of total RNA was used for reverse transcription (33) by using the oligo(dT) primer (Invitrogen). The resulting cDNA was stored frozen (−20°C) until use.

PCR conditions. Cytokine-specific oligonucleotide primer pair sequences for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (5′-CCACCC ATGGCAAATTCCATGGCA-3′ and 5′-TCTAGACGGCAGGTCAGGTCCA CC-3′), MMP-2 (5′-GTGCTGAAGGACACACTAAAGAAGA-3′ and 5′-TTG CCATCCTTCTCAAAGTTGTAGG-3′), MMP-9 (5′-CACTGTCCACCCCTC AGAGC-3′ and 5′-GCCACTTGTCGGCGATAAGG-3′), and TIMP-1 (5′-AT CCTGTTGTTGCTGTGGCTGATAG-3′ and 5′-TGCTGGGTGGTAACTCTT TATTTCA-3′) were obtained from Invitrogen. Samples were amplified by use of a DNA thermocycler (Perkin-Elmer Cetus, Emeryville, CA), as previously described (33), during 25 cycles (94°C for 45 s, 60°C for 45 s, and 72°C for 90 s) for GAPDH and 30 cycles for MMPs and TIMP-1. PCR products were visualized on 1.7% agarose gels, and the specificity of the amplified bands was validated by their predicted sizes (GAPDH, 600 bp; MMP-2, 618 bp; MMP-9, 382 bp; TIMP-1, 630 bp). Densitometry analysis was performed by scanning the images from the agarose gels (video documenting system; Amersham Pharmacia), and values were obtained via ImageMaster software (Pharmacia). Each experiment included a negative control to which no cDNA was added. Serial samples from a given patient were all assayed in parallel.

Real-time PCR. For quantitative PCR analysis, amplification was carried out by using 100 ng cDNA added to tubes in triplicate containing TaqMan gene expression assay mixtures for the detection of interleukin-10 (IL-10), IFN-γ, TNF-α, MMP-9, and TIMP-1 (Applied Biosystems, Foster City, CA) via the ABI Prism 7000 sequence detection system (Applied Biosystems), as previously described (41). The relative expressions of the target messages were continuously compared during the log phase of PCR in terms of the ΔC_T (mean threshold cycle [C_T] of 3 replicate experimental sample cDNAs minus the mean C_T of 3 replicate GAPDH cDNAs).

Gelatin zymography. When skin biopsy specimens were used for the zymography procedures, the dermis was homogenized in the Politron apparatus in 1 ml of culture medium (RPMI medium), and the homogenates were stored at −80°C until use. Samples to be processed for zymography (culture supernatants and homogenized biopsy specimens) were recovered, loaded onto a 15% SDS-PAGE gel containing 1 mg/ml gelatin (Sigma Chemical Co., St. Louis, MO), and processed, as described above (28). Briefly, the gels were sequentially treated in 2.5% Triton X-100 for 30 min, followed by an overnight incubation at 37°C in a gelatinase substrate buffer (50 mM Tris-HCl, 10 mM $CaCl_2$, 0.02% NaN_3 [pH 8.0]), and stained with 0.5% Coomassie blue. The quantification of gelatinase levels was achieved by scanning densitometry (Amersham Pharmacia).

***In situ* zymography.** To determine whether proteolytic activity was present in skin tissue, *in situ* zymography was performed on unfixed frozen sections using a gelatin substrate. This technique employed fluorescein isothiocyanate (FITC)-labeled gelatin (Molecular Probes, Eugene, OR), the proteinase substrate that loses its fluorescent signal when cleaved. Unfixed frozen sections of tissue were incubated with reaction buffer (0.05 M Tris-HCl, 0.15 M NaCl, 5 mM $CaCl_2$, and 0.2 mM NaN_3 [pH 7.5]) containing 30 mg/ml gelatin for 48 h. These are optimal conditions for detecting MMP activity, but activity due to other neutral proteinases could also be detected. At the end of the incubation period, fluorescence was observed by using a light microscope equipped for epifluorescence (Nikon E400), and images were captured via a digital camera. The same sequential sections were used as negative controls to which EDTA was added in a reaction buffer to inhibit gelatinase activity.

MMP-9 and TIMP-1 ELISA. MMP-9 and TIMP-1 levels in serum samples and supernatants of *M. leprae*-stimulated PMBC were measured by means of an enzyme immunoassay with paired antibodies. The MMP-9 and TIMP-1 assays used reagents from R&D Systems. Plates were coated with mouse anti-MMP-9 and TIMP-1, and the detection antibody from the Quantikine kit (DMP900) was used. This assay detects total MMP-9, both the proform and the active form.

Statistical analysis. Results are reported as pooled data from an entire series of experiments and for each group of individuals and are shown as means ± standard errors of the means (SEM). For data comparisons, the Mann-Whitney, Kruskal-Wallis, and two-way analysis of variance (ANOVA) tests were used. The correlation between the level of expression of MMPs and the level of expression of cytokines was evaluated for each group of patients (MB and PB) via the Spearman test. The statistical significance level adopted was a P value of <0.05.

RESULTS

MMP-2 and MMP-9 expression and activity are enhanced in the dermis of tuberculoid versus lepromatous lesions. An evaluation of the relative amounts of cytokines and MMP transcripts was performed by using skin samples from tuberculoid leprosy (T-lep group; $n = 6$) and lepromatous leprosy (L-lep group; $n = 6$) patients (Fig. 1A). The level of expression of TNF-α and IFN-γ mRNAs in the T-lep dermis was significantly higher than that in the L-lep dermis ($P < 0.05$). Similarly, levels of expression of the MMP-2 and MMP-9 messages were also higher ($P < 0.05$ and $P < 0.01$, respectively) in T-lep lesions. On the other hand, TIMP-1 and IL-10 message expression levels did not change significantly under either of the 2 clinical conditions. The association between the expression of MMPs and the IFN-γ and TNF-α cytokines was also evaluated for each group of patients. In the T-lep (paucibacillary [PB]) group, the correlations between MMP-9 and TNF-α and between MMP-9 and IFN-γ were found to be significant ($P < 0.01$). With respect to MMP-2, a correlation was observed only between this gelatinase and TNF ($P < 0.05$). Within the L-lep group (MB), while all these correlations were possible, none were statistically significant ($P > 0.05$).

MMPs are proteases that must be cleaved to be activated. The detection of enzyme secretion cannot determine the activation state of MMPs, and the difference in molecular mass between pro-MMP-9 and active MMP-9 is only 7 kDa. To determine gelatinolytic activity in leprosy lesions, zymography was performed with the tissue homogenates (Fig. 1B). Even though the characteristic MMP-2 (72-kDa) and MMP-9 (92-kDa) bands were detected, the lower-molecular-mass bands with gelatinolytic activity could not be seen. Following densitometry analysis of the gel bands and in accordance with RNA data, the level of detection of MMP-2 and MMP-9 was found to be higher in the T-lep dermis than in the L-lep dermis. In addition, *in situ* zymography was used to assess skin sections to demonstrate the localization of MMP activity. As shown in Fig. 1C, gelatinase activity (represented by a focal reduction in fluorescence) detected *in situ* in the T-lep lesions was found to be concentrated in the granuloma structures (Fig. 1C, right), whereas in the control sections, no change in the fluorescence pattern was observed (Fig. 1C, left). Furthermore, no gelatinase activity was detected in the L-lep lesions (not shown).

MMP-2 and MMP-9 mRNA activities increase in the dermis during leprosy reactions. The levels of expression of MMP-2 and MMP-9 and their tissue inhibitor TIMP-1 in the dermis of 18 reactional patients (8 RR and 10 ENL patients were assayed by reverse transcription-PCR (RT-PCR). MMP-2 and MMP-9 mRNA levels were higher than TIMP-1 levels in both forms of reaction (Fig. 2A). For ENL patients, differences were significant between MMP-2 and MMP-9 versus TIMP-1 ($P < 0.01$), whereas for the RR group, significance was noted only between MMP-9 and TIMP-1 ($P < 0.01$) (Fig. 2A). Interestingly, the extents of expression of MMPs and TIMP-1 detected between ENL and RR were not different, which is indicative of their role in triggering tissue damage under both conditions.

It was also of interest to evaluate the expression of MMPs in sequential biopsy specimens taken from the same patient before and at the onset of a reaction. A total of 7 patients (3 RR and 4 ENL patients) were assayed, and real-time PCR was performed simultaneously for all samples obtained from each patient. As shown in Fig. 2B, the relative amounts of TNF-α, MMP-2, and MMP-9 mRNAs were enhanced ($P < 0.05$) during RR (left) and ENL (right) as opposed to before reaction (BR). However, TIMP-1 message levels decreased during RR and varied insignificantly during ENL in comparison to what their levels were prior to the reaction ($P < 0.05$).

To evaluate MMP activation in reactional lesions, zymography was performed on extracts from dermis samples of 2 reactional patients prior to and during reactions, revealing the presence of bands corresponding to pro-MMP-2 (72 kDa) and pro-MMP-9 (92 kDa), which is indicative of dermal gelatinase production in the skin of leprosy patients (Fig. 2C, left). More interestingly, the active MMP-2 (65-kDa) and MMP-9 (82-kDa) forms, absent in BR, were observed in the dermal reactional samples, which may be due to intense MMP activity during these inflammatory episodes. Densitometry scanning of the gel confirmed the prevalence of MMP-9 and MMP-2 activity detected in the skin lesions prior to and during reactions. By use of *in situ* zymography (Fig. 2D), gelatinolytic activity was located inside the inflammatory infiltrate in RR lesions, as demonstrated by the absence of fluorescent gelatin degradation (Fig. 2D, right), similar to what was observed for the T-lep lesions (Fig. 1C).

MMP-2 and MMP-9 are differentially modulated in leprosy skin lesions. The levels of expression of MMP-2 and MMP-9 were then evaluated by immunohistochemistry and confocal microscopy (Fig. 3 and 4, respectively). In T-lep lesions ($n =$

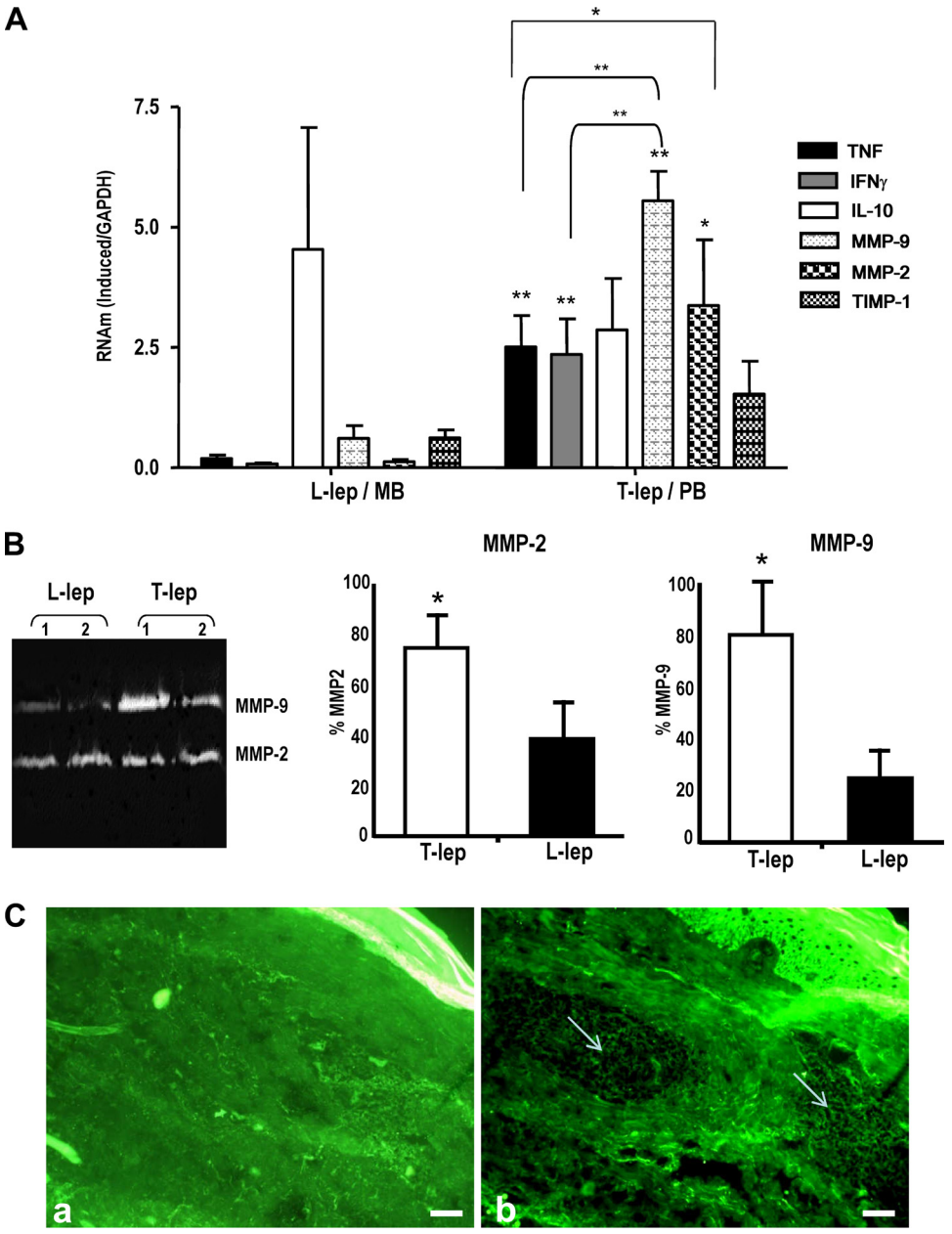

FIG. 1. Expression of MMPs and cytokines and MMP activity in leprosy skin. Biopsy specimens were taken from lepromatous (L-lep) (n = 6) and tuberculoid (T-lep) (n = 6) lesions. Real-time PCR was performed to evaluate the levels of expression of TNF-α, IFN-γ, IL-10, MMP-9, MMP-2, and TIMP-1 mRNAs. Results are the relative expression levels of the target message presented as means ± SEM for each group of patients tested. The levels of expression of MMPs and the cytokines but not TIMP-1 were significantly enhanced in the T-lep group compared to the L-lep group. (B) Gelatin zymography was performed on dermis samples from 3 L-lep and 3 T-lep patients. Bands shown in the gel (representative of 2 patients in each group) correspond to the characteristic 72-kDa MMP-2 and 92-kDa MMP-9. Densitometry analysis was performed, and values in the graph are pooled data for all patients tested. (C) MMP gelatinolytic activity in leprosy skin lesion samples was assayed by *in situ* zymography. For T-lep patients, gelatinolytic activity was identified as the dark areas (arrows) in the inflammatory infiltrate (b) compared to the negative control sections to which EDTA was added (a), and no protein activity was observed. Scale bar, 50 μm. *, $P < 0.05$; **, $P < 0.01$.

4), positive staining was concentrated in the granuloma cell components, although focal epidermal positivity was also observed (Fig. 3A). The positivity of MMP-9 seen in L-lep skin samples (n = 2) was mildly dispersed in the dermis (Fig. 3B), whereas immunostaining for TIMP-1 showed slight and diffuse positivity, making a descriptive analysis difficult (not shown). Confocal microscopy performed on 3 patient samples (T-lep lesions) (Fig. 4A to C) showed a colocalization of MMP-9 with CD68, confirming that macrophages were the main source of MMP-9 production in these lesions (Fig. 4C). As for the L-lep lesions (n = 2), it cannot be ruled out that dermal fibroblasts and endothelial cells (Fig. 4D to F) were positive since no

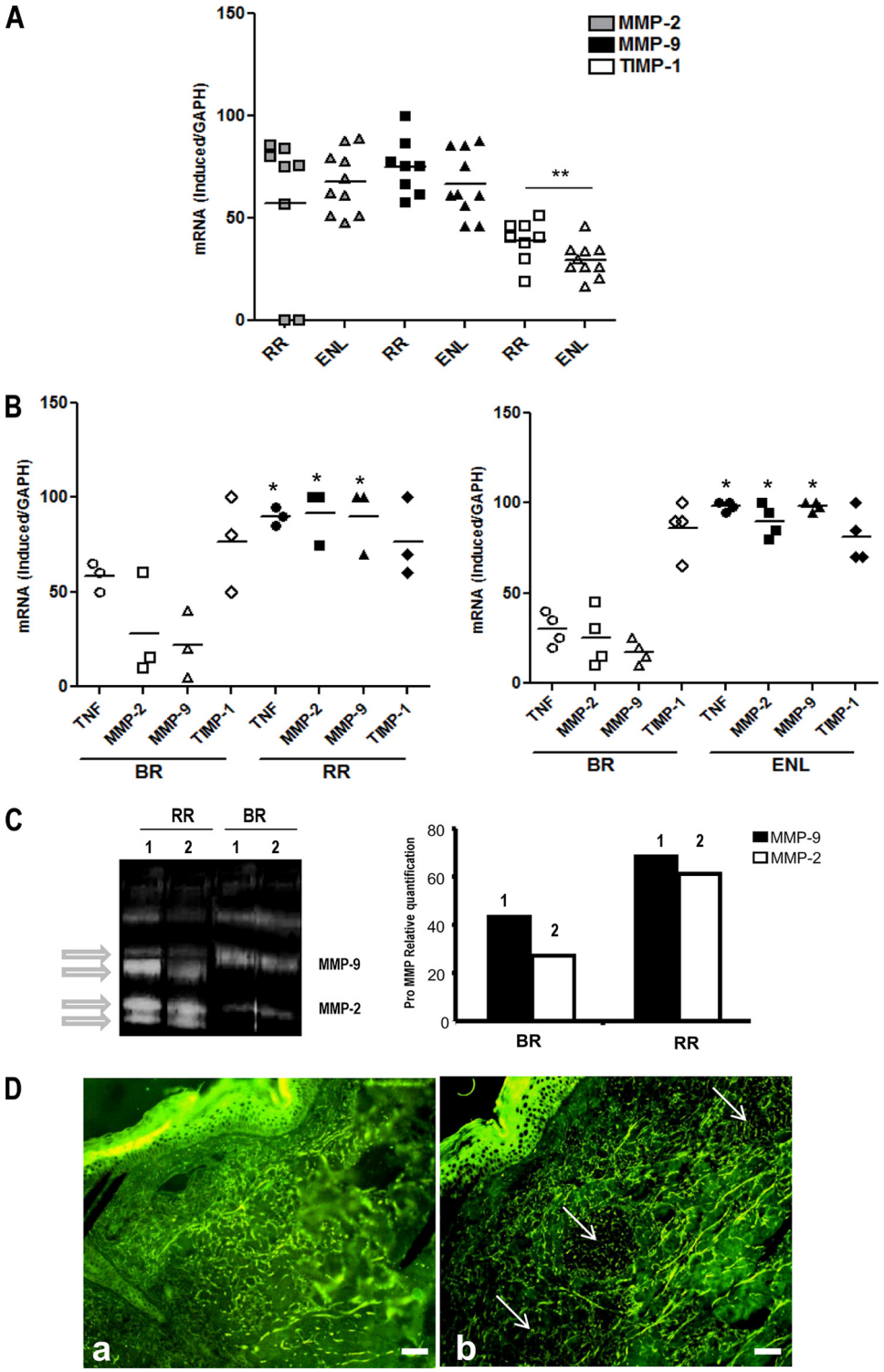

FIG. 2. Detection of MMP mRNA and protein activity in leprosy reactions. MMP and TIMP-1 mRNA levels in reactional skin lesions were assayed by RT-PCR. (A) MMP-2 and MMP-9 mRNA levels were higher than those of TIMP-1 in both forms of reaction (ENL, $n = 10$; RR, $n = 8$). No differences were detected between ENL and RR, but significant differences were seen between MMP-2 and MMP-9 in comparison with TIMP-1 in ENL and between MMP-9 and TIMP-1 in the RR group (**, $P < 0.01$). (B) Evaluation of TNF-α, MMP, and TIMP-1 mRNAs in skin lesions obtained from the same patient and assayed before reaction (BR) and during reaction (3 RR and 4 ENL patients). The mRNA level was significantly enhanced during reaction in the same patient compared to that before reaction, except for TIMP-1, the level of which decreased during RR (*, $P < 0.05$). (C) Zymography evaluation of MMP-9 protein activity in reactional lesions (RR, 2 patients) showed bands that corresponded to pro-MMP-2 and pro-MMP-9 in the dermis before and during reactions. Data in the graph represent data from densitometry analyses of the gel. (D) *In situ* zymography identified gelatinolytic activity in RR skin lesions in the dark areas (arrows) of the tissue inflammatory infiltrate (b), which was absent in the negative control sections (a). Scale bar, 50 μm.

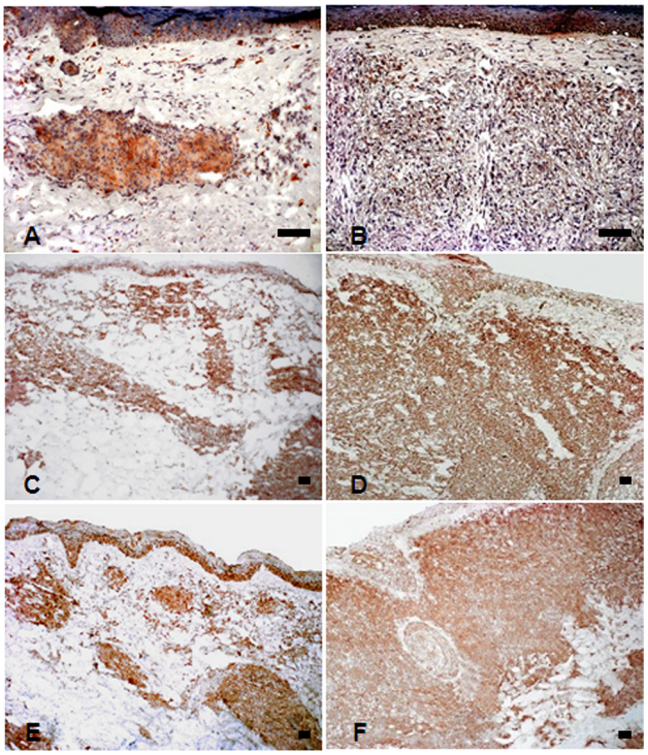

FIG. 3. MMP-2 and MMP-9 expression levels in leprosy skin lesions. Immunostaining of MMPs was performed in cryostat sections, visualized by the immunoperoxidase method, and counterstained with hematoxylin. (A) In T-lep lesions, MMP-9 positivity was concentrated inside the granuloma and was also detected in the focal areas in keratinocytes. (B) In L-lep skin, MMP-9 was only mildly expressed, demonstrating a disperse distribution in the inflammatory infiltrate. (C and E) In RR, positive MMP-2 (C) and MMP-9 (E) staining was limited to the inflammatory infiltrate. The epidermal basal layer is almost continuously positive. (D and F) In ENL, dense MMP-2 staining (D) and, on the other hand, diffuse MMP-9 (F) are superimposed onto the inflammatory infiltrate occupying the whole dermis. MMP-2 was also detected in the epidermis, while MMP-9 was not. Images are representative of each group of patients tested (BT = 4; LL = 2; RR = 3; ENL = 2). Scale bars, 50 μm.

predominant colocalization with CD68 was observed (Fig. 4F). The MMP-9 and CD68 colocalization percentage rate observed for T-lep skin was 19%, and that for L-lep skin was 3%.

Immunohistochemistry analysis of MMPs assayed in reactional lesions demonstrated strong positivity, corresponding to the inflammatory infiltrate in both RR and ENL (Fig. 3C to F). The presence of focal MMP positivity (MMP-2 [Fig. 3C] andMMP-9 [Fig. 3E]) was detected in the epidermis of RR lesions, in contrast to the absence of positivity in the ENL epidermis (MMP-2 [Fig. 3D] and MMP-9 [Fig. 3F]). Moreover, while in RR (Fig. 3C and E), MMP staining in the skin was found to be concentrated in the granuloma (similarly to T-lep lesions), in ENL, the lesions showed intense positivity, completely covering the inflammatory infiltrate (Fig. 3D and F). In these lesions, not only macrophages but also other cell types, including granulocytes, could be MMP producers.

Increased MMP-9 serum levels were detected in sera from reactional patients. To verify the systemic release of MMP-9 and TIMP-1 into peripheral blood, serum samples from 12

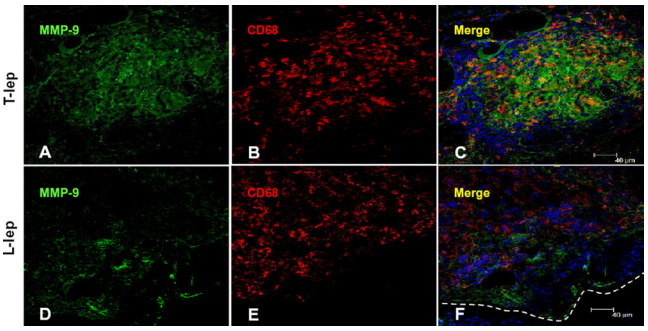

FIG. 4. Coexpression of MMP-9 and CD68 detected in leprosy skin samples. (A to C) Confocal microscopy was performed with patient samples (T-lep = 3; L-lep = 2), and immunostaining for MMP-9 (A) and CD68 (B) in samples from T-lep patients showed MMP-9 colocalizing with inflammatory macrophages ($CD68^+$ cells) in the granuloma (C); the colocalization of MMP-9 (green; Alexa 488) and CD68 (red; Alexa 568) is indicated by orange staining. (D to F) MMP-9 (D) and CD68 (E) staining in the L-lep lesions did not show any colocalization (F). DAPI blue staining indicates the nuclei, and the dotted line indicates the dermal-epidermal limit. Images were visualized and captured by use of a confocal microscope (Leica) and are representative of each group of patients tested. Scale bar, 40 μm.

unreactional patients (L-lep, $n = 7$; T-lep, $n = 5$), 11 reactional patients (ENL, $n = 6$; RR, $n = 5$), and 4 healthy controls were collected and measured by ELISA. Figure 5 shows that MMP-9 levels were significantly higher in both L-lep (169 ± 29.3 ng/ml; $P < 0.001$) and T-lep (205 ± 29 ng/ml; $P < 0.01$) reactional and unreactional patients. In the ENL group, an elevated serum level of MMP-9 (563 ± 52.2 ng/ml), but not TIMP-1, was observed ($P < 0.05$). The highest MMP-9 serum levels detected in ENL were higher than the values found for the control group (389 ± 84 ng/ml; $P < 0.05$). While not significant, the same profile was noted for RR patients. In contrast, among L-lep patients, TIMP-1 values (407.2 ± 82

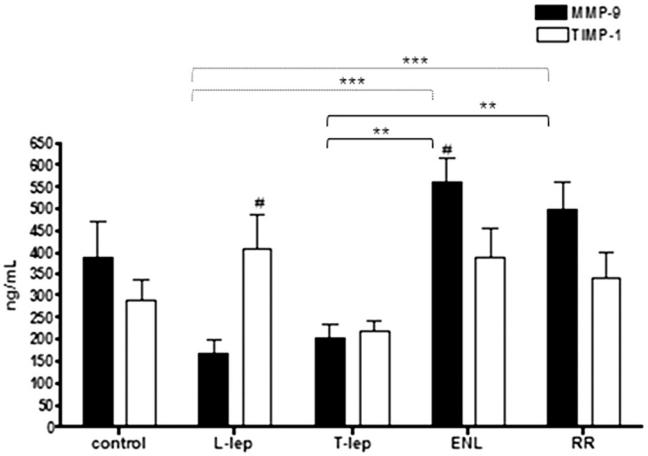

FIG. 5. MMP-9 and TIMP-1 detected in patient sera. Sera were obtained from 12 unreactional (7 T-lep and 5 L-lep) and 11 reactional (5 RR and 6 ENL) patients in addition to 4 healthy donors (controls), all evaluated by ELISA. Results represent means ± SEM. The Kruskal-Wallis test was used to compare the different groups (**, $P < 0.01$; ***, $P < 0.001$; *, $P < 0.05$ [compared to the control group]). Two-way ANOVA was used to compare MMP-9 and TIMP-1 levels within the same group (#, $P < 0.05$).

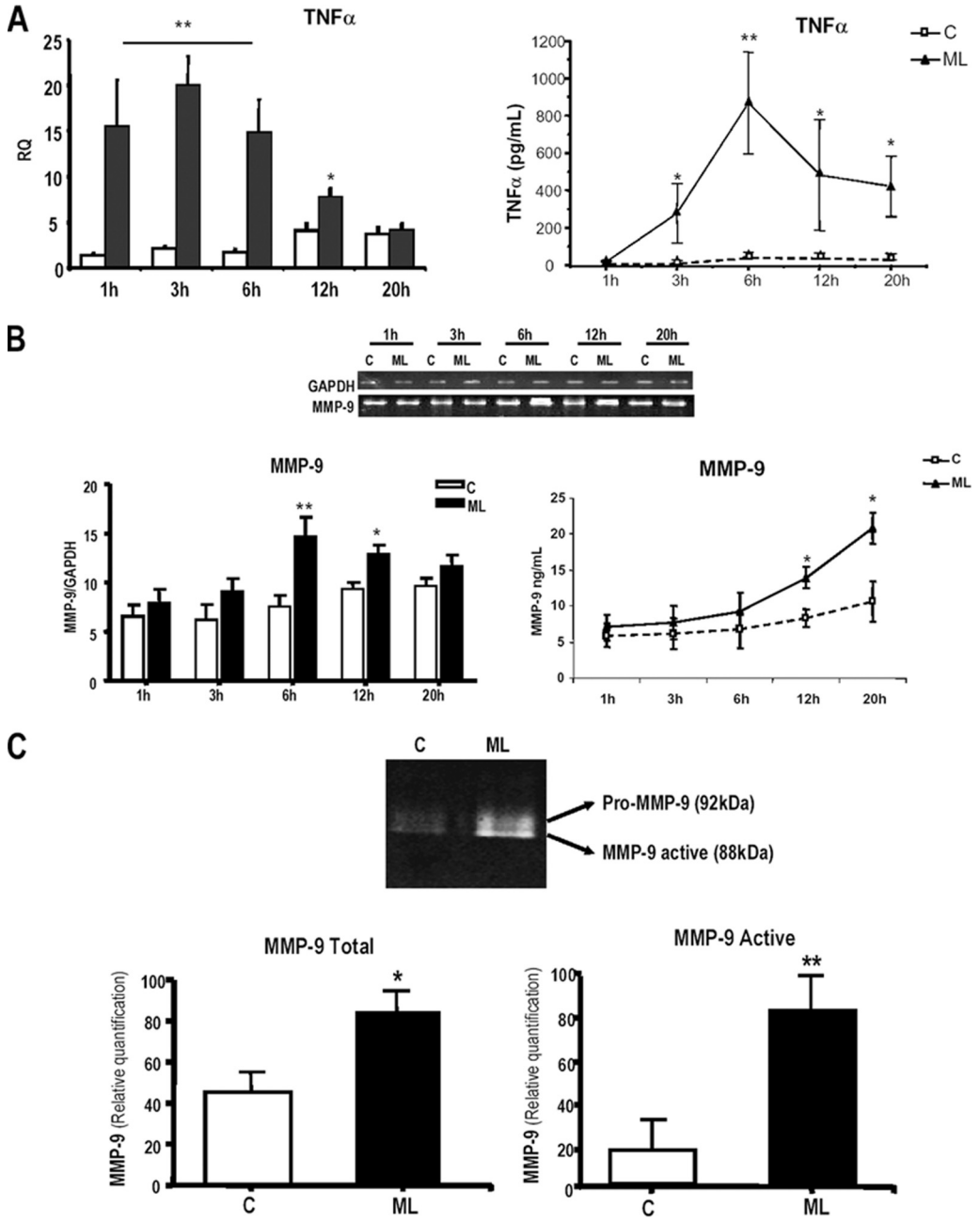

FIG. 6. *M. leprae* induces MMP-9 expression in PBMC *in vitro*. PBMC obtained from L-lep patients, both unstimulated (control [C]) and stimulated with *M. leprae* (ML) (10 μg/ml), were assayed after different culture periods. (A) TNF-α mRNA was evaluated by real-time PCR (left), and protein levels were assessed by ELISA (right). The relative quantification (RQ) of TNF-α message induced by *M. leprae* already showed enhanced expression after 1 h of culture. (B) Evaluation of MMP-9 mRNA (left) and protein (right) levels indicated a peak of the response at 6 and 20 h, respectively. (C) MMP-9 activity measured by zymography showed representative bands in the supernatants of the PBMC cultures corresponding to pro-MMP-9 (92 kDa) and active MMP-9 (88 kDa). Densitometry scanning of the gel confirmed the predominance of MMP-9 induced by *M. leprae* versus the control cells. Results are means (±SEM) for data from 7 independent experiments (*, $P < 0.05$; **, $P < 0.01$).

ng/ml) were higher than those of MMP-9 (169 ± 29.3 ng/ml; $P < 0.05$).

***M. leprae* induces MMP-9 expression and activity in PBMC of leprosy patients.** Analysis of the clinical specimens described so far indicates that MMP-2 and MMP-9 are being produced *in situ* during the course of the disease. To determine whether *M. leprae* is able to induce MMP-9 production, as was previously reported for other mycobacteria (12, 28), PBMC from leprosy patients ($n = 7$) were stimulated *in vitro* with *M. leprae* after different culture periods, and mRNA and protein expression levels were evaluated as described above. Following *M. leprae* stimulation (10 μg/ml), the induction of the TNF-α message was already highly upregulated after 1 and 3 h of culture ($P < 0.01$) (Fig. 6A). TNF-α secretion in the culture

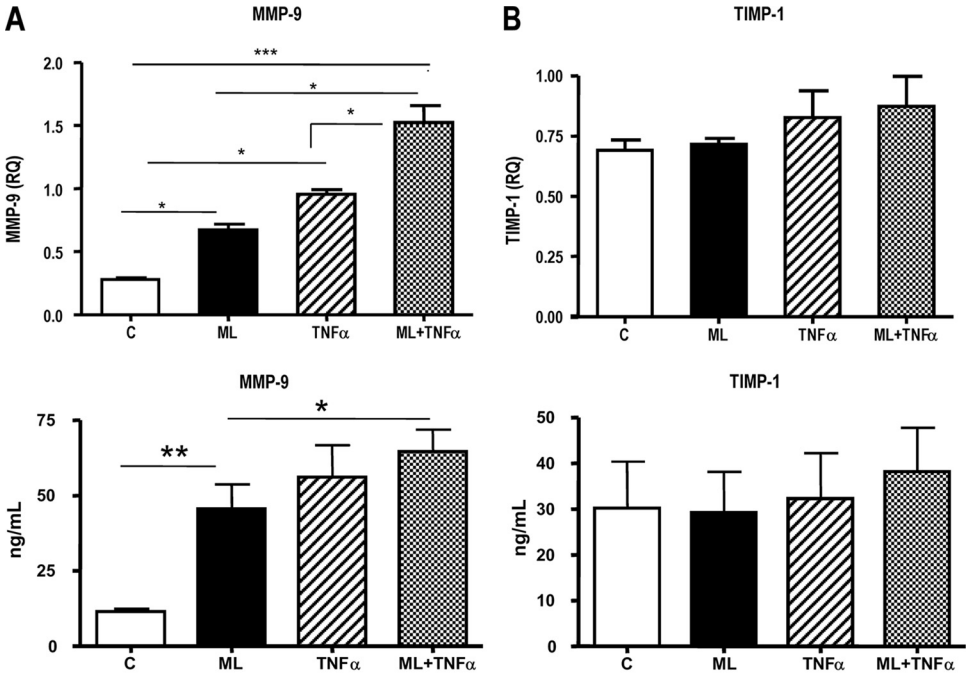

FIG. 7. *M. leprae* and TNF-α induce MMP-9 production *in vitro*. Shown are data for PBMC from L-lep patients, both unstimulated (control [C]) and stimulated with *M. leprae* (ML) (10 μg/ml), TNF-α (10 ng/ml), and *M. leprae* plus TNF-α for 6 h (mRNA) and 24 h (protein release). MMP-9 (A) and TIMP-1 (B) mRNA (top) and protein (bottom) levels were assayed by real-time PCR and ELISA, respectively. The values represent means ± SEM ($n = 5$). Statistical differences were evaluated by the Mann-Whitney test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

supernatants reached a peak after 6 h ($P < 0.01$). The level of expression of MMP-9 mRNA was seen 3 h after the addition of the mycobacteria, and it was even higher after 6 h ($P < 0.01$) and 12 h ($P < 0.05$) (Fig. 6B). The ability of *M. leprae* to induce MMP was also observed for the PBMC obtained from healthy donors (not shown). The induction of MMP-9 was enhanced after 6 h, and levels were higher still after 20 h ($P < 0.05$) (Fig. 6B). For the same cultures, no modification of TIMP-1 or MMP-2 occurred during the experiments (not shown).

To determine whether both pro-MMP-9 and active MMP-9 can be detected in *M. leprae*-stimulated cultures, zymography was performed on the supernatants of PBMC stimulated or not with the mycobacteria (24-h cultures). The visualization of the bands in the gel in Fig. 6C corresponds to both pro-MMP-9 (92 kDa) and active MMP-9 (88 kDa) following *M. leprae* activation. Pooled densitometry analysis from the different experiments demonstrated a significant induction of MMP-9 ($P = 0.02$) in comparison to the control supernatants (Fig. 6C). Overall, the data confirm the ability of *M. leprae* to induce the expression of the active forms of MMP-9.

TNF synergizes with *M. leprae* in the induction of MMP-9 in PBMC cultures. Since TNF-α was previously shown to promote MMP secretion (32, 35), we then investigated whether *M. leprae* and TNF-α could drive MMP production *in vitro*. Cells from 5 L-lep patients were stimulated with mycobacteria (10 μg/ml), TNF-α (10 ng/ml), or *M. leprae* and TNF-α for 6 and 24 h, when MMP-9 and TIMP-1 production was evaluated, as described above. As shown in Fig. 7A (top), the MMP-9 mRNA level increased in the presence of TNF-α ($P < 0.05$) and *M. leprae* ($P < 0.05$) in addition to *M. leprae* and TNF-α ($P < 0.001$) in comparison with the control cells. Moreover, increased levels of MMP-9 mRNA were detected when *M. leprae*- and TNF-α-stimulated cells were compared with *M. leprae*- or TNF-α-stimulated cultures ($P < 0.05$). With regard to protein release, the MMP-9 levels induced by *M. leprae* and TNF in culture supernatants (64.5 ± 7.3 ng/ml) were higher than those induced by *M. leprae* alone (45 ± 8 ng/ml) but not significantly different from the levels induced by TNF alone (56 ± 10.6 ng/ml) (Fig. 7A, bottom). For TIMP-1 (Fig. 7B), although its levels were slightly elevated in comparison to those of *M. leprae* alone, the variation in mRNA expression induced by *M. leprae* plus TNF was insignificant ($P > 0.05$) (bottom). Moreover, protein levels were not altered by either *M. leprae* or TNF (Fig. 7B, bottom).

DISCUSSION

Reactional episodes are very intriguing events occurring during the course of leprosy, an infectious disease. Although both innate immunity and adaptive immunity against the pathogen are involved in the specific mechanisms leading to skin and nerve damage in both RR and ENL, the precise components are not completely understood. In the present study, higher MMP-2 mRNA, MMP-9 mRNA, and protein levels were detected together with enhanced TNF-α and IFN-γ expression levels in the lesions of tuberculoid patients and in those undergoing a reaction. MMP proteolytic activity depends not only on the number of transcripts but also on the degree of molecular cleavage in the membrane itself, together with the action of specific inhibitor molecules present in the tissue environment. In summary, our data evidenced a positive MMP/TIMP ratio induced by *M. leprae* infection during the inflammatory process both *in vivo*

and *in vitro*. By use of *in situ* zymography, local MMP activity in both tuberculoid and reactional skin lesions associated with the granuloma infiltrates was confirmed.

During a reaction, patients suddenly present with an inflammatory infiltrate that invades the skin abruptly as a result of TH1 cytokine release. Due to immune cell migration, IFN-γ and TNF-α are upregulated, and an enhanced expression of MMPs was evidenced *in situ*. In this scenario, the heterogeneous clinical and histopathological presentations ranging from localized to extensive and/or necrotic lesions suggested that both the tissue environment and remodeling play a crucial role in the clinical presentation of skin damage.

The cytokines associated with $CD4^+$ T-cell subpopulations may influence MMP production in a different manner. TH2 cytokines like IL-4 and IL-10 were previously shown to predominate in lepromatous leprosy lesions (19, 22) and to downregulate MMP-9 (4). However, the effects of TH1 cytokines are not as clear. IL-15 and IL-18 are able to upregulate MMP-9 in macrophages (1), while, conversely, the effects of IFN-γ are predominantly inhibitory (14, 17). In experimental models of mycobacterial infection, the addition of IFN-γ inhibits MMP-9 production by peritoneal macrophages (28). In contrast, IFN-γ was shown previously to synergize with IL-1β to upregulate MMP-9 activity in *M. tuberculosis*-infected monocytes (11).

In tuberculosis (TB), the upregulation of MMP-9 was correlated with the widespread tissue destruction that is a hallmark of this disease (37). In leprosy, however, tissue destruction occurs only during a reaction. It is noteworthy that in tuberculoid leprosy lesions, they do not promote tissue proteolysis in the skin even when granulomas are abundant, in contrast to what occurs with TB, suggesting a tight balance between proteolytic enzymes and their regulatory systems. Moreover, in TB granuloma, MMP-9 positivity is localized around the necrotic area (37). In leprosy (both in tuberculoid and RR lesions), MMPs are localized in the central area of the granuloma, in which macrophages and epithelioid cells are predominant. Necrosis is rarely seen in T-lep lesions even though caseous abscesses are a frequent finding in nerve lesions, suggesting that in the skin, MMP regulatory mechanisms are more efficient at balancing their proteolytic activity.

To our knowledge, this is the first time that the ability of *M. leprae* to induce MMP expression in blood mononuclear cells has been demonstrated. The induction of MMP was found to be preceded by TNF-α transcription and release, suggesting that, *in vitro*, the production of MMP-9 could be mediated by TNF-α. The addition of TNF to the cultures increased the levels of MMP production, reinforcing this hypothesis. TNF-α induces MMP-9 expression in many systems (24, 38), while MMP-9 has the ability to activate TNF-α release from its membrane precursor (43). Endogenous TNF-α stimulates MMP-9 gene transcription in monocytes through NF-κB activation (3), and p38 mitogen-activated protein kinase (MAPK) inhibitors were able to downregulate MMP-9 mRNA and protein expression (24). Therefore, inhibitors of MAPK pathways could benefit from the damage caused by excessive inflammation.

A serum MMP-9 and MMP-9/TIMP-1 imbalance may be a risk factor for triggering inflammatory processes. The detection of systemic MMPs has been used broadly for correlations with disease severity for many inflammatory conditions such as rheumatoid arthritis, type 2 diabetes, multiple sclerosis, lupus erythematosus, as well as cancer (7, 26, 31) and may have great value in disease diagnosis and prognosis. However, comparative research should be carried out with caution since the results often seem to be overly influenced by the collection and processing techniques adopted (15). TIMPs inhibit the active MMP forms by specifically binding with the catalytic site, and they were also reported to sometimes inhibit the latent forms as well. Therefore, any disturbance in this balance may lead to tissue damage. TIMP-1 is more capable of binding with the active forms, especially that of MMP-9, but can also bind to active MMP-2 (13, 23, 39, 40).

In this study, the MMP-9 levels detected in patient sera correlated with the increased level of expression of this protein in reactional lesions. The low TIMP-1 levels seem to become imbalanced with MMP expression when disseminated leukocyte migration is in course during a reaction. Similarly, in lepromatous patients, lower MMP-9 levels in sera correlated with the low cell turnover reported for these lesions and could be related to sustained TIMP-1 levels. Unexpectedly, the systemic MMP-9 levels in tuberculoid patients were similar to those found for the lepromatous group. Nevertheless, TIMP-1 levels were significantly more elevated in the latter group.

The systemic response observed herein seems to correlate with the imbalance in proteolytic activity at the lesion site in the T-lep and reactional groups. Moreover, the highest serum levels of MMP-9 detected in ENL correlated with the clinical and histopathological findings whenever disseminated inflammatory lesions, characterized by intense macrophage and granulocytic infiltrates, appeared all over the body. The intense positivity seen via immunohistochemistry confirmed these data. Conversely, some studies have shown that augmented synthesis and MMP-9 secretion are induced via cytokines, mainly TNF-α, secreted by activated macrophages present in the inflammatory infiltrates (35).

In this study, a large number of clinical specimens taken from stable, untreated leprosy lesions during a reaction confirmed participation of MMPs in the disease. Higher mRNA levels in skin biopsy specimens correlated with the expression of the proinflammatory cytokines TNF-α and IFN-γ. On the other hand, the detection of protein in the inflammatory infiltrate correlated with tissue damage and increased levels of MMPs in the sera during the reaction, which was especially true for ENL. Furthermore, the induction of MMP-9 by *M. leprae* alone seemed to be reinforced by the addition of TNF-α. Overall, these results pave the way for new therapeutic interventions during leprosy reactions.

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