

# LL-37 Immunomodulatory Activity during *Mycobacterium tuberculosis* Infection in Macrophages

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Tuberculosis is one of the most important infectious diseases worldwide. The susceptibility to this disease depends to a great extent on the innate immune response against mycobacteria. Host defense peptides (HDP) are one of the first barriers to counteract infection. Cathelicidin (LL-37) is an HDP that has many immunomodulatory effects besides its weak antimicrobial activity. Despite advances in the study of the innate immune response in tuberculosis, the immunological role of LL-37 during *M. tuberculosis* infection has not been clarified. Monocyte-derived macrophages were infected with *M. tuberculosis* strain H37Rv and then treated with 1, 5, or 15  $\mu$ g/ml of exogenous LL-37 for 4, 8, and 24 h. Exogenous LL-37 decreased tumor necrosis factor alpha (TNF- $\alpha$ ) and interleukin-17 (IL-17) while inducing anti-inflammatory IL-10 and transforming growth factor  $\beta$  (TGF- $\beta$ ) production. Interestingly, the decreased production of anti-inflammatory cytokines did not reduce antimycobacterial activity. These results are consistent with the concept that LL-37 can modulate the expression of cytokines during mycobacterial infection and this activity was independent of the P2X7 receptor. Thus, LL-37 modulates the response of macrophages during infection, controlling the expression of proinflammatory and anti-inflammatory cytokines.

**T**uberculosis (TB), caused by *Mycobacterium tuberculosis*, is the single deadliest communicable disease. In 2013, an estimated 9.0 million people developed TB and 1.5 million died from the disease (1).

The main cells involved in the control of tuberculosis are macrophages, and together with epithelial cells, they are the first antiinfective immunological barriers encountered, with a primary task to initiate pathogen clearance. In the progress of cellular immunity against M. tuberculosis, macrophages can also function as antigen-presenting cells, in which the antigens of M. tuberculosis are degraded into immunogenic polypeptides and presented on T lymphocytes by the major histocompatibility complex to trigger adaptive immunity. However, M. tuberculosis has developed a wide assortment of strategies to counteract the bactericidal activities of these cells, enabling it to successfully establish a niche for long-term survival within macrophages. This M. tuberculosis replication causes mild inflammation, which promotes cell-mediated immunity that often leads to M. tuberculosis retention through granuloma (tubercle) formation (2). When infection becomes reactivated at a low rate, the granuloma suffers caseous necrosis, and this results in lung cavitation and pulmonary disease, inducing a prominent inflammation (3). Several molecules of the immune system are involved throughout this process, including host defense peptides (HDP) such as cathelicidin and defensins (4-6).

LL-37 is the unique member of cathelicidin family in humans; this multifunctional immunomodulatory HDP is produced mainly by phagocytic leukocytes and epithelial cells as well as being normally found at concentrations varying from 2 to 5  $\mu$ g/ml in several fluids and tissues; however, this concentration increases during inflammatory processes, including those associated with infections (7). Besides its weak antimicrobial activity, LL-37 has both proinflammatory and anti-inflammatory immunomodulatory effects (8). Thus, it seems that LL-37 can selectively modulate host immune responses to enable the resolution of pathogen-induced inflammation while maintaining or enhancing anti-infective immunity. The mechanisms used by LL-37 to achieve this modulation are complex; however, it has been demonstrated that LL-37 targets inflammatory pathways such as Toll-like receptor to NF-kB in the presence of either pathogenic or immune-mediated inflammatory stimuli, resulting in selective suppression of proinflammatory responses, while maintaining or enhancing critical immune responses such as cell recruitment (9). The suppression of specific-pathogen-induced proinflammatory responses, such as the induction of tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), TNF- $\alpha$ -induced protein-2, matrix metalloproteinase 3 (MMP-3), and nitric oxide, occurs in part through the enhancement of the production of TNF- $\alpha$ -induced protein-3, NF-KB inhibitor NFKBIA, and expression of IL-10 and the IL-1 antagonist (IL-1RA) (9-12). Moreover, this human cathelicidin modulates gamma interferon (IFN- $\gamma$ ) responses during both innate and adaptive phases of the immune response, promoting an anti-inflammatory milieu (7). Studies have suggested that in intracellular uptake it is important for the immunomodulatory activity of LL-37 (8, 13), and this peptide also interacts with multiple cell surface receptors such as FPRL-1, CCR6, and P2X7, mediating different events depending on the cell type and perhaps the exog-

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enous stimuli. Nevertheless, it has not been determined whether the inflammasome-linked receptor P2X7 contributes to cathelicidin anti-inflammatory activity.

Several studies have shown the importance of LL-37 in containing M. tuberculosis growth during the early stages of the infection (4, 14, 15). Furthermore, our group determined the kinetics of cathelicidin expression during M. tuberculosis infection in a mouse experimental model, showing a high cathelicidin expression in three peaks: the first peak observed very early after 1 day of infection, a second one at day 21, when the peak of protective immunity in this model was achieved, and a third peak (associated with strong cathelicidin immunostaining in vacuolated macrophages filled with bacilli) observed at day 60 postinfection, when advanced progressive disease was well established and characterized by high bacillary loads and extensive tissue damage (16). This strong expression in vacuolated macrophages suggests that cathelicidin might have greater immunomodulatory effects than antimicrobial activity during advanced disease, consistent with the known suppression of cathelicidin activity under physiological conditions. Thus, cathelicidin may have a dual function; during early infection, it might be an important factor, expressed by lung epithelial cells and alveolar macrophages, that contributes to the control of mycobacterial growth, and during advanced progressive disease it could be a significant immunomodulatory factor, perhaps in suppressing excessive inflammation.

This report provides evidence that LL-37 promotes anti-inflammatory cytokines in *M. tuberculosis*-infected macrophages.

#### MATERIALS AND METHODS

*M. tuberculosis* culture. Drug-sensitive *M. tuberculosis* strain H37Rv (ATCC 27294, Manassas, VA, USA) was cultured in 25-cm<sup>2</sup> plastic culture flasks with 10 ml of Middlebrook 7H9 broth (Difco, Detroit, MI, USA) supplemented with 0.2% (vol/vol) glycerol, 10% oleic acid, albumin, dextrose, and catalase (OADC enrichment medium; BBL, Becton Dickinson, Franklin Lakes, NJ) and incubated at 37°C with 5% CO<sub>2</sub> atmosphere until the bacteria reached the logarithmic phase of growth, which was determined by daily measurements of the optical density at 600 nm (OD<sub>600</sub>). Once determined to be in the log phase, the culture was divided into working aliquots of  $2 \times 10^8$  cells/ml and frozen at  $-80^\circ$ C until use. For experiments involving heat-killed mycobacteria, a 1,000-µl vial containing  $1 \times 10^9$  bacilli was submerged (using a lead weight) in a water bath preheated and maintained at 80°C for 2 h, after which the mycobacteria were washed three times with phosphate-buffered saline (PBS) and plated onto a 7H10 agar plate to confirm inactivation.

Cell preparation and infection. The study was approved by the National Committee of Ethics and National Commission of Scientific Research of the Mexican Institute of Social Security (IMSS). The study was performed according to the Declaration of Helsinki. After written informed consent was obtained, subjects underwent venipuncture, and heparinized blood was obtained from the 6 purified protein derivative (PPD)-negative healthy donors, none of them having a history of prior exposure to TB patients. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (Nycomed Pharma AS, Oslo, Norway). PBMC were cultured in RPMI medium using 24-well dishes (Costar Ontario, Canada) or chamber slides (Costar, Corning, NY). After 2 h, nonadherent cells were removed. The remaining adherent cells were washed at least three times with Hanks' balanced salt solution (BioWhittaker, Walkersville, MD). Cells were incubated with homologous serum, and after 7 days, monocyte-derived macrophages (MDMs) were used for infection. Cytospin preparations were prepared from adherent uninfected cells to allow evaluation of the nuclear and cellular morphology by Wright's staining. The purity of MDMs was assessed by flow cytometry (>90%).

The human U937 promonocytic cell line was obtained from American

Type Culture Collection (ATCC) and maintained in RPMI 1640, supplemented with 10% fetal bovine serum (FBS; Sigma) and 5,000 units/ml penicillin. The cells were incubated in an atmosphere of 5% CO<sub>2</sub> at 37°C. All cells used in this study were between passages 5 and 15. Viability was checked by the Guava Viacount Assay (Millipore, Billerica, MA, USA) showing >95% of viability. Cells were cultured in 24-well dishes or in chamber slides and incubated overnight under 5% CO<sub>2</sub> at 37°C with 1 ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis, MO, USA) to transform U937 cells into an adherent macrophagelike state (MDMs), supernatants were removed the next day, and the adherent cells were washed thoroughly with Hanks' salt solution.

The *in vitro* infection was done as previously reported (4). Briefly, MDMs either from healthy donors or from U937 cells were plated separately in 24-well dishes at a concentration of  $5 \times 10^5$  cells per well, in addition to plating in 4-well chamber slides. The cells were infected with *M. tuberculosis* in RPMI 1640 supplemented with 30% of non-heat-inactivated pooled human AB serum. MDMs were left untreated (medium alone) or infected at a multiplicity of infection (MOI) of 5:1, and then cells were incubated at 37°C in a 5% CO<sub>2</sub> atmosphere for 2 h to allow phagocytosis, and the nonphagocytosed mycobacteria were removed through vigorous washing with Hanks' solution supplemented with streptomycin.

For stimulations, infected (after 2 h of infection, when bacilli were phagocytosed) or uninfected cells were incubated at  $37^{\circ}$ C and 5% CO<sub>2</sub> with 1, 5, or 15 µg/ml of LL-37 for 4, 8, and 24 h as described previously (17). Then, supernatant was collected and stored at  $-70^{\circ}$ C in the presence of protease inhibitor or 200 µl TRIzol (Life Technologies, Inc., Gaithersburg, MD) until use. Before RNA isolation, cell culture viability was checked, showing 99% viability. To abrogate P2X7 activity, we used an anti-P2X7 monoclonal antibody (Abcam, Cambridge, MA).

**Phagocytosis assays.** To determine whether MDMs from the U937 cell line and those from healthy donors were comparable, we assessed the percentage of phagocytosis and calculated a phagocytosis index. MDMs  $(5 \times 10^5 \text{ cells/ml})$  were cultured and adhered into 4 well-chamber slides (Lab Tek) and infected as described above for 2 h, and then cells were vigorously washed with Hanks' solution to eliminate nonphagocytosed mycobacteria. Cells were fixed and stained with the conventional Ziehl-Neelsen (ZN) method. To determine the percentage of phagocytosis, at least 3,000 cells were counted and those cells with at least one mycobacterium inside were counted as positive. To determine the phagocytosis index, in the cells that were positive for phagocytosis, the phagocytosed bacilli per infected cell were counted. Experiments were done in triplicate in three independent experiments.

Intracellular *M. tuberculosis* growth with exogenous LL-37. After infection with *M. tuberculosis* as described above, we wanted to determine whether the exogenous LL-37 would improve the capacity of the MDMs to kill mycobacteria; several concentrations of LL-37 (1, 5, or 15  $\mu$ g/ml) were separately added to the infected MDMs and incubated for 24 h at 37°C, 5% CO<sub>2</sub>. Then, cells were lysed with SDS, serial dilutions were plated in triplicate onto Middlebrook 7H10 agar (Difco), and CFU were determined after 21 days of incubation at 37°C, 5% CO<sub>2</sub>. In representative experiments, the viability of the cell cultures was assessed at 12 and 24 h postinfection by Trypan blue exclusion.

The mean concentrations of the frozen H37Rv stock suspensions were determined by counting CFU on 7H10 agar plates in triplicate serial dilutions of unclumped stock suspensions between days 21 and 28. This unclumping procedure was performed in each experiment to ensure that we were using single-bacterial-cell suspensions and to establish the input amounts of bacteria for the infections at the various MOIs.

**RNA isolation and RT.** Reverse transcription (RT) of mRNA was performed using 5  $\mu$ g RNA, 2  $\mu$ M oligo(dT) 15 primer (Promega, Ontario, Canada), 10 units RNase inhibitor (10 units/ $\mu$ l; Invitrogen, Carlsbad, CA), 1× RT buffer, 0.5 mM each deoxynucleoside triphosphate (dNTP), and 4 units Omniscript reverse transcriptase (Qiagen, Inc., Mexico). Realtime PCR was performed using a Light Cycler 2.0 (Roche, Germany), Light Cycler TaqMan Mastermix, and the specific probe for each gene that

Gene encoding:	Sequence		
	Probe	Forward primer	Reverse primer
IL-1β	TTC CTG GC	TCT TTG GGT AAT TTT TGG GAT CT	TAC CTG TCC TGC GTG TTG AA
TNF-α	CTT CTG CC	GCC AGA GGG CTG ATT AGA GA	CAG CCT CTT CTC CTT CCT GAT
IL-10	CTG GGG CT	CAT AAA TTA GAG GTC TCC AAA ATC G	AAG GGG CTG GGT CAG CTA T
TGF-β HPRT	AGC TGG AG GCT GAG GA	CAG CCG GTT GCT GAG GTA CGA GCA AGA CGT TCA GTC CT	GCA GCA CGT GGA GCT GTA TGA CCT TGA TTA TTT TGC ATA CC

TABLE 1 Sequence of primers and TaqMan probes used for RT-qPCR assays<sup>a</sup>

<sup>*a*</sup> Abbreviations: IL-1β, interleukin-1β; TNF-α, tumor necrosis factor alpha; IL-10, interleukin-10; TGF-β, transforming growth factor β; HPRT, hypoxanthine-guanine phosphoribosyltransferase; RT-qPCR, real-time quantitative PCR.

had been designed using the Universal Probe Library software (Roche, Germany) (Table 1). The relative expression of each sample was calculated using human hypoxanthine-guanine phosphoribosyltransferase (HPRT) mRNA as a reference gene and the  $2^{-\Delta\Delta CT}$  method (where  $C_T$  is threshold cycle) as described previously (18). This method was based on the expression levels of a target gene compared to a reference gene (*HPRT*), comparing between control group and target group.

Flow cytometry assay. To determine surface markers,  $1 \times 10^{6}$  MDM cells uninfected or infected with *M. tuberculosis* were stained with various antibodies. An anti-HLA-DR allophycocyanin (APC)-labeled antibody (BD Biosciences, San Diego, CA, USA) was used to determine the maturation state of the MDMs. For P2X7 receptor determinations in MDMs, a monoclonal rabbit anti-human antibody (Abcam, Cambridge, United Kingdom) was used together with a goat anti-rabbit phycocrythrin (PE)-labeled antibody (Santacruz Biotechnology, Dallas, TX, USA) as a second staining step. Cells were analyzed in a FACS-CANTO II flow cytometer using the FACS DIVA software, (where FACS is fluorescence-activated cell sorter) v6.1.3 for analysis (Becton Dickinson, San Jose, CA, USA).

Cytokine determinations by CBAs. Supernatant cytokine concentrations for IL-10, TNF- $\alpha$ , IL-1 $\beta$  (mature form), IL-6, IL-8, IL-17, transforming growth factor  $\beta$  (TGF- $\beta$ ), and IFN- $\gamma$  were measured using the BD cytometric bead array (CBA) human Flex Set system (BD Biosciences, San Diego, CA, USA). For these determinations, 50  $\mu$ l from each cell supernatant 1 was used. Fluorescence intensity (FI) from the immunoassay was measured using a BD FACS-CANTO II cytometry system and the FACS DIVA v6.1.3, and for the data analysis the BD FCAP Array Software v3.0.1 (BD Biosciences, San Diego, CA, USA) was used according to the manufacturer's instructions.

**Statistical analysis.** Statistical analyses were performed using the GraphPad Prism software for Mac (GraphPad Software version 6.01, San Diego, CA). Normal distribution was assessed using the Kolmogorov-Smirnov test for each data set, together with a nonparametric two-group comparison U-Mann-Whitney or multiple-comparison Kruskal-Wallis test to identify differences among the groups. When statistical significance (P < 0.05) was found, a Dunn's posttest was performed. Two-sided P values of <0.05 were considered statistically significant.

#### RESULTS

Differences between HD-MDMs and U937 MDMs. To evaluate whether MDMs from U937 were a suitable model for our studies, we first compared the percentage and index of phagocytosis for MDMs from healthy donors (HD-MDMs) with those for U937 MDMs. Our results showed that there were no differences either for the percentage of phagocytosis (Fig. 1A) or for the phagocytosis index (Fig. 1B) between HD-MDMs and U937 MDMs. To assess whether there were statistical differences with respect to viability in the presence of different concentrations of LL-37, cells were stimulated for up to 24 h with different concentrations of LL-37, showing viability of >90% for 15  $\mu$ g/ml, >95% for 5  $\mu$ g/ ml, and >98% for 1  $\mu$ g/ml (Fig. 1C). Subsequently, we evaluated the percentages of HLA-DR-positive cells in both kinds of cells, given that this cell surface marker correlates with MDM maturation. Furthermore, we determined whether the different stimuli shifted HLA-DR expression between cells. Thus, cells were stimulated with 5  $\mu$ g/ml of LL-37 for 8 h or infected with *M. tuberculosis* at an MOI of 5 for 2 h, and a third set of cells were infected for 2 h with *M. tuberculosis* and then stimulated for 8 h with 5  $\mu$ g/ml of LL-37 (Fig. 1D). Since no statistical differences were found between the two groups of cells and due to the logistic difficulty in recruiting PPD-negative subjects, we decided to work with U937-MDMs, referred to here as MDMs.

LL-37 shifts TNF- $\alpha$  expression in *M. tuberculosis*-infected macrophages. To assess the influence of LL-37 on inflammatory activity in *M. tuberculosis*-infected MDMs, IL-1 $\beta$  and TNF- $\alpha$ 



FIG 1 U937 MDMs do not differ significantly from donor MDMs. (A and B) Percentage (A) and index (B) of phagocytosis of MDMs from healthy patients (HD-MDM) and the U937 cell line (U937-MDM) were determined by infecting MDMs with *M. tuberculosis* at an MOI of 5:1 for 2 h, after which cells were fixed and stained with ZN. (C) MDMs were exposed for 24 h to different concentrations of LL-37 (1, 5, and 15 µg/ml) to find out if LL-37 affects viability of HD-MDMs in the absence (Control) or presence of *M. tuberculosis* at an MOI of 5:1 were incubated with or without LL-37 (5 µg/ml) for 8 h to find out if HLA-DR expression changed. There were no changes between cell models. Data are presented as medians with interquartile ranges. In each experimental group, n = 6.



FIG 2 Effects of LL-37 on TNF-α and IL-1β mRNA expression in *M. tuber-culosis*-infected macrophages (Mtb). MDMs in the absence (Control) or presence of *M. tuberculosis* at an MOI of 5:1 were incubated with LL-37 (1, 5, and 15 µg/ml) at 37°C and 5% CO<sub>2</sub> for 4, 8, and 24 h. The relative mRNA expression of IL-1β was determined at 4 h (A), 8 h (B), and 24 h (C) after stimulation. We observed better induction at 4 and 24 h in infected cells treated with LL-37. Expression of TNF-α increased at 4 h (D) and decreased at 8 (E) and 24 (F) hours in all concentrations of LL-37 added to infected cells. Data are presented as medians with interquartile ranges. In each experimental group, n = 6. \*\*, P < 0.01; \*\*\*, P < 0.001 compared with *M. tuberculosis*-infected macrophages.

mRNA expressions were evaluated several times after stimulation with different LL-37 concentrations. Our results showed that 5  $\mu$ g of LL-37 alone did not induce abundant production of these cytokines, except for TNF- $\alpha$ -production after 24 h of stimulation. In *M. tuberculosis*-infected cells, without LL-37 treatment, we observed an increase in TNF- $\alpha$  as early as 8 h.

Interestingly, adding 1, 5, and 15  $\mu$ g/ml of the cathelicidin to infected cells substantially increased the IL-1 $\beta$  gene expression mainly at 4 h (Fig. 2A) and at 24 h (Fig. 2C) poststimulation; however, at 8 h poststimulation (Fig. 2B) IL-1 $\beta$  modestly increased about 15-fold. When TNF- $\alpha$  was evaluated at 4 h, *M. tuberculosis* with LL-37 induced the upregulation of this gene (Fig. 2D). In contrast, at 8 and 24 h, LL-37 potently downregulated *M.* 

*tuberculosis*-induced TNF- $\alpha$  (Fig. 2E and D, respectively), whereas LL-37 and *M. tuberculosis* alone separately induced its upregulation. P2X7 blocking showed no statistical differences compared with similar conditions without blocking antibody. When we used heat-killed *M. tuberculosis* to infect MDMs, both IL-1 $\beta$  and TNF- $\alpha$  had no statistical differences compared with noninfected cells (see Fig. 4A and C, respectively). Our data demonstrated that LL-37 induces IL-1 $\beta$  gene expression whereas it downregulates TNF- $\alpha$  in *M. tuberculosis*-infected MDMs, and these phenomena are P2X7 independent.

LL-37 upregulated IL-10 and TGF-B in M. tuberculosis-infected macrophages. To further explore the immunomodulatory role of cathelicidin in infected macrophages, we determined the expression of 2 important anti-inflammatory cytokines produced during progressive tuberculosis: IL-10 and TGF-B (19). Our results showed that both cytokines were upregulated when M. tuberculosis-infected macrophages were treated with different concentrations of cathelicidin at 4 h (Fig. 3A and D), 8 h (Fig. 3B and E), and 24 h (Fig. 3C and F). The fold change of treated infected MDMs varied from 5 to 30 compared to the control and infected MDMs alone for both cytokines. LL-37 or M. tuberculosis alone induced a marked downregulation of IL-10, while a modest downregulation or no change was seen in TGF-B expression. Similar experiments using heat-killed M. tuberculosis showed that LL-37 did not induce gene upregulation either for TGF- $\beta$  or for IL-10 (Fig. 4B and D, respectively). In summary, results showed that LL-37 induced TGF- $\beta$  and IL-10 mRNA upregulation in *M*. tuberculosis-infected cells, and this effect can be seen only in cells infected with viable bacteria.

Effects of LL-37 on cytokine response in M. tuberculosis-infected macrophages. Results from the mRNA expression analysis suggested that LL-37 anti-inflammatory activity was first detected after 2 h postinfection and with stimulation with 5 µg/ml of LL-37 for 8 h as described above. To confirm this, the concentrations of several cytokines were measured in the cell supernatants at this time, except for IL-10, for which the concentration was measured at 48 h postinfection, due to the fact that this cytokine has a delayed expression. Results for TNF- $\alpha$  and IL-1 $\beta$  correlated with those found in mRNA expression analysis, showing that exogenous LL-37 reduced TNF- $\alpha$  production in comparison with *M*. tuberculosis-infected cells alone, and conversely, exogenous LL-37 in M. tuberculosis-infected cells modestly increased the production of IL-1B (Fig. 5A and B, respectively). Regarding IL-10 and TGF-B, exogenous cathelicidin treatment of infected macrophages increased the production of both cytokines 5-fold compared with nontreated infected cells (Fig. 5C and D).

A crucial role for IL-17 has been described in the generation and recruitment of neutrophils in response to inflammation and infection; this cytokine is produced mainly by a special lymphocyte subset and macrophages (20); thus, we determined whether LL-37 promoted the production of this proinflammatory cytokine in infected macrophages. Our results showed that exogenous LL-37 decreased the production of IL-17 compared with infected macrophages alone (Fig. 5E). We also explored other cytokines that are involved in the immunopathogenesis of tuberculosis and are produced by macrophages such as IL-6, IFN- $\gamma$ , and IL-8 (21). Our results showed no statistical differences between *M. tuberculosis*-infected cells and cells infected and then stimulated with LL-37 (Fig. 5F, G, and H). Given that exogenous LL-37 promoted an anti-inflammatory milieu, we sought to determine whether this



**FIG 3** Effects of LL-37 on IL-10 and TGF-β mRNA expression in *M. tuberculosis*-infected macrophages. The mRNA expression of anti-inflammatory cytokines such as IL-10 and TGF-β was determined by real-time PCR. MDMs in the absence (Control) or presence of *M. tuberculosis* at an MOI of 5:1 were treated with LL-37 (1, 5, and 15 µg/ml). LL-37 increased the expression of mRNA IL-10 at 4 (A), 8 (B), and 24 (C) hours on infected cells. In like manner, mRNA TGF-β increased at 4 (D), 8 (E), and 24 (F) hours. The data are presented as medians with interquartile ranges. In each experimental group, *n* = 6. \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001 compared with *M. tuberculosis*-infected macrophages.

condition affected the antimycobacterial activity of MDMs in our model. Thus, we added different concentrations of LL-37 to infected MDMs, and after 24 h the *M. tuberculosis* killing was evaluated by CFU. Results showed that CFU decreased in a dose-dependent manner, suggesting that though LL-37 plays an immunomodulatory role, it also could be uptaken by infected cells to eliminate bacteria (Fig. 6).

**LL-37 anti-inflammatory activity in** *M. tuberculosis*-infected cells did not lead to downregulation of P2X7. Previous studies have shown the participation of the nucleotide scavenging receptor P2X7 in inflammatory activities induced by LL-37. Thus, we hypothesized that *M. tuberculosis* phagocytosis would downregulate P2X7 levels on the cell surface, thus promoting the LL-37



FIG 4 Proinflammatory/anti-inflammatory cytokine expression is not affected in heat-killed *M. tuberculosis*-infected macrophages (HK-Mtb) treated with LL-37. MDMs were infected with heat-killed *M. tuberculosis* (MOI, 5:1) and then treated with 5 µg/ml of LL-37 for 8 h. mRNA relative expression was measured for IL-1β (A), TGF β (B), TNF- $\alpha$  (C), and IL-10 (D). No statistical differences were found between groups. The data are presented as medians with interquartile ranges. In each experimental group, n = 3.

anti-inflammatory activity that has been described above. Infected HLA-DR<sup>+</sup> MDMs (Fig. 7A) were gated for P2X7 (Fig. 7B). Results showed that neither LL-37 nor *M. tuberculosis* infection changed the percentage of P2X7<sup>+</sup> cells (Fig. 7C). Likewise, the mean fluorescence intensity (MFI) values of P2X7-positive cells were similar for the different stimuli (Fig. 7D), suggesting that neither *M. tuberculosis* nor LL-37 alone or added as an exogenous peptide affected P2X7 expression; thus, the immunoregulatory function reported in the present study is P2X7 independent.

## DISCUSSION

Cathelicidin is a very important HDP that has been suggested to be involved in the control of tuberculosis mainly through its direct antimicrobial activity. Studies have demonstrated its ability to promote the elimination of *M. tuberculosis*, showing that the main cells that produce the cathelicidin during infection are alveolar macrophages and MDMs (4, 14, 15). Our group found in an experimental model of pulmonary tuberculosis during advanced progressive disease (characterized by high bacillary loads and extensive tissue damage) a strong immunostaining of cathelicidin in highly infected vacuolated macrophages, which constitute a significant source of anti-inflammatory/immunosuppressive molecules such as TGF- $\beta$ , prostaglandin E, and IL-10 (16, 19, 22). On the other hand, it has been demonstrated that LL-37 inhibits cellular responses to IFN- $\gamma$  (7), the key cytokine of Th1-polarized immunity and antimycobacterial activity. The strong expression of this HDP in vacuolated macrophages suggests that LL-37 might have immunosuppressive effects during advanced disease. Intriguingly, other studies have shown that LL-37 in the presence of macrophage colony-stimulating factor (M-CSF) and without the



FIG 5 Effects of LL-37 on proinflammatory/anti-inflammatory cytokines in *M. tuberculosis*-infected macrophages. Cytokine concentrations were determined by CBAs; MDMs uninfected (Control) or infected with *M. tuberculosis* at an MOI of 5:1 for 2 h were stimulated with LL-37 (5 µg/ml) for 8 h. Administration of LL-37 decreases the concentration of TNF- $\alpha$  (A) and IL-17 (E) in infected cells. Anti-inflammatory cytokines IL-10 (C) and TGF- $\beta$  (D) were increased when stimulated with LL-37. Other cytokines like IL-1 $\beta$  (B), IL-6 (F), IFN- $\gamma$  (G), and IL-8 (H) do not show statistical differences. The data are presented as medians with interquartile ranges. In each experimental group, *n* = 6. \*, *P* < 0.05; \*\*, *P* < 0.001; \*\*\*, *P* < 0.001.

presence of any microorganism directs macrophage differentiation toward macrophages with a proinflammatory signature (23). To evaluate the cathelicidin immunomodulatory activity during *M. tuberculosis* infection, we first assessed whether the cells in our U937-MDM model were similar to MDMs from PPD-negative (*M. tuberculosis*-uninfected) healthy donors (HD-MDMs). Results showed that there were no differences between the two groups of cells and none of the concentrations of LL-37 used significantly reduced cellular viability. Regarding cell maturation, there were no differences between the two cell types, including several conditions such as infection and exogenous cathelicidin. Thus, these results indicated that U937-MDMs and HD-MDMs had no differences that might interfere with the interpretation of our results; therefore, and due to the difficulty of recruiting PPDnegative donors, we performed this study with U937-MDMs. Subsequently, we showed that all LL-37 concentrations used in this study promoted IL-1 $\beta$  mRNA expression in infected macrophages, even though the protein concentrations in the supernatants remained the same. Previous studies have demonstrated that LL-37 alone or during *M. tuberculosis* infection induces IL-1 $\beta$ mRNA expression in MDMs and subsequently caspase-1 activation by P2X7/inflammasome leads to IL-1 $\beta$  maturation. In our model, it is possible that infection led to P2X7/inflammasome malfunctioning, thus leading to the decreased production of matured IL-1 $\beta$ .

As expected, TNF- $\alpha$  levels were increased when cells were infected with *M. tuberculosis* or stimulated with LL-37. Interestingly, in infected macrophages treated with cathelicidin, TNF- $\alpha$  levels decreased substantially after 8 and 24 h of stimulation. Similar studies described that LL-37 also inhibits the IFN- $\gamma$  priming of



FIG 6 Bacillary loads decreased in an LL-37 dose-dependent manner in infected MDMs. To assess whether upregulation of anti-inflammatory cytokines correlates with increasing bacillary loads, MDMs were infected with *M. tuberculosis* (MOI, 5:1) as described previously and then treated with different LL-37 concentrations for 24 h. MDMs were lysed, and intracellular bacilli were plated onto 7H10 agar plates and incubated for 21 days at 37°C. CFUs were determined and graphed. The data are presented as medians with interquartile ranges. In each experimental group, n = 6. \*, P < 0.05.

lipopolysaccharide (LPS) responses and the synergistic response to a combined treatment with IFN- $\gamma$  and LPS, suggesting an important modulatory function of this peptide not only promoting inflammation but balancing the response to avoid exacerbated inflammation.

To assess the immunosuppressive role of the cathelicidin in M. tuberculosis-infected macrophages, we measured IL-10 and TGF-β levels; both mRNA expression levels and protein concentration showed that exogenous LL-37 increased substantially these cytokines in infected macrophages, and interestingly, IL-17 levels decreased significantly, suggesting a strong anti-inflammatory activity. In active TB disease, inflammation increases; susceptible hosts with active TB disease maintain a high degree of inflammation and develop consumption, frequently resulting in a fatal outcome. Inflammatory factors thus contribute to pathogen persistence, dissemination, and transmission. Soluble and cellular proinflammatory effectors not only promote antimicrobial functions but also orchestrate the accumulation of M. tuberculosispermissive cells interfering with regulatory pathways and inducing extensive tissue injury, and thus it is plausible that exogenous LL-37 may play a balancing role to avoid exacerbated inflammation, promoting anti-inflammatory and blocking proinflammatory cytokines. It has been described that the ability of some individuals to produce properly LL-37 confers a certain degree of resistance to M. tuberculosis infection whereas individuals that do not produce sufficient amounts of cathelicidin might be prone to develop tuberculosis (15, 24). This effect has been attributed to LL-37 direct antimicrobial activity during early infection; the results of this study suggest that cathelicidin immunomodulatory activity may also confer protection during the progress of infection, avoiding excessive inflammation and bacillus dissemination, promoting an anti-inflammatory milieu and direct antimicrobial activity as well. It is probable that individuals lacking proper LL-37 production because of genetic alterations or vitamin D deficiency not only are susceptible to primary infection but also are not capable of modulating inflammation, thus leading to disease progression with an uncontrolled inflammation whose frequent outcome is pulmonary dysfunction; however, this issue must be further studied in detail.

The mechanisms that mediate the LL-37 anti-inflammatory activity in *M. tuberculosis*-infected MDMs have not been studied.



FIG 7 P2X7 expression is not altered during *M. tuberculosis* infection. Expression of surface markers was determined by flow cytometry. MDM cells (1 × 10<sup>6</sup>) were stimulated with LL-37 (5 µg/ml), *M. tuberculosis* (MOI, 5:1), or both and then were analyzed for expression of HLA-DR and P2X7. The histograms represent cells' HLA-DR (A) and P2X7 (B). The percentage of positive cells to HLA-DR (C) was determined in MDMs as well as MFI of P2X7-positive cells (D). Neither LL-37 nor *M. tuberculosis* infection changed the percentage of P2X7<sup>+</sup> cells. The data are presented as medians with interquartile ranges. In each experimental group, n = 6.

It has been reported that LL-37 is an endogenous ligand for P2X7, which is a robust inducer of inflammasome activation in macrophages and monocytes. Stimulation of this receptor by LL-37 enhances COX2 expression and prostaglandin E2, besides triggering the secretion of several proinflammatory substances, such as IL-1 $\beta$ , IL-18, TNF- $\alpha$ , and nitric oxide (25, 26). We hypothesized that M. tuberculosis phagocytosis would downregulate P2X7 levels on the cell surface and the interaction between receptor and ligand could thus be abrogated, therefore decreasing inflammation cytokines. However, our results showed that there were no changes of P2X7 expression during *M. tuberculosis* phagocytosis; similarly, exogenous LL-37 did not induce any significant change. These data suggest that P2X7 is not involved in the process of immunomodulation dependent on LL-37 during tuberculosis progression. When we blocked P2X7 with a P2X7-blocking antibody in infected MDMs treated with exogenous LL-37, there were no significant differences regarding cytokine mRNA expression, suggesting that P2X7 is not involved in the immunomodulation seen in the present study. Other studies have demonstrated that suppressive effects of LL-37 on IFN- $\gamma$  responses were mediated through the inhibition of STAT1-independent signaling events, involving both the p65 subunit of NF-KB and p38 mitogen-activated protein kinase (MAPK) (7). Whether this or another mechanism is involved in the LL-37 immunomodulation process during tuberculosis is unknown and needs to be further studied.

Interestingly, when we infected cells with heat-killed mycobacteria, none of the immunomodulatory effects were seen; this result suggests that immunoregulation is generated by an *M. tuberculosis*-secreted antigen. Previously, it has been reported that the 30and 38-kDa antigens (Ag85B complex) produced by H37Rv increase IL-10 expression, although other secretion antigens such as ESAT-6 induce similar responses (27, 28); it is plausible that these antigens are necessary to induce LL-37 modulatory effects; however, further research is needed to specifically identify the responsible antigen. Thus, we can hypothesize that not all *M. tuberculosis* strains might induce the same immunomodulatory effect; this will depend on the quantity and sort of antigen that each strain produces.

It has been reported that patients with active pulmonary tuberculosis have an alveolar inflammation resulting in the release of TNF- $\alpha$  and IL-1 $\beta$  in bronchoalveolar epithelial fluid. It was proposed that the levels of these cytokines would correlate with clinical status parameters and that their naturally occurring inhibitors would be concomitantly released in the local inflammatory sites (29). The soluble forms of TNF- $\alpha$  receptors have inhibitory properties against TNF- $\alpha$ ; on the other hand, the IL-1 soluble receptor binds to IL-1ß during progressive tuberculosis and may serve to suppress the inflammatory consequences of early-released IL-1ß (30). Together, these soluble receptors promote an anti-inflammatory milieu during pulmonary tuberculosis; however, in patients with severe pulmonary tuberculosis these receptor levels are downregulated and not correlated with TNF- $\alpha$  and IL-1 $\beta$  levels or with disease status (29). We hypothesize that LL-37 might be involved in the induction of both receptors, promoting an antiinflammatory response. Conversely, patients with active tuberculosis despite having high LL-37 levels (31, 32) still develop an extended pneumonia; this fact prompts us to propose that LL-37 levels in these patients are not as high as thought previously and correlates with previous studies in which individuals with low LL-37 production are more prone to develop tuberculosis (15); therefore, once the pneumonia develops, they will not be able to induce a proper anti-inflammatory milieu, leading to exacerbated pneumonia. However, further investigations are needed to elucidate this issue.

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