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Antimicrobial and immunomodulatory activity induced by loperamide in mycobacterial infections

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ABSTRACT

Loperamide is an antidiarrheal drug that targets μ-opioid receptors and calcium channels. A previous report demonstrated that loperamide induces autophagy and enhances antimicrobial activity towards *M. tuberculosis* in murine and human alveolar macrophages. The aim of this study was to evaluate the immunomodulatory effects of loperamide on macrophages with respect to cytokine and antimicrobial peptide production during mycobacterial infection. We infected monocyte-derived macrophages (macrophages) with *M. tuberculosis* H37Rv at a multiplicity of infection (MOI) of 5 and treated the cells with 3 μM loperamide. Cytokine production in the supernatants of 24-h cultures and gene expression of the cytokines TNFα, IL1β and IL10 and the antimicrobial peptides LL37 and bactericidal/permeability increasing protein (BPI) in the cell lysates was measured. Intracellular bacterial loads were evaluated by enumerating colony-forming units 3 days posttreatment for *M. tuberculosis* and 24 h posttreatment for *M. smegmatis*. We observed that loperamide exerted an immunomodulatory effect on TNFα production in human macrophages infected with *M. tuberculosis* and that these responses were independent of the bacteria, as they also occurred when macrophages were infected with *M. smegmatis* and to a lesser extent with *M. bovis*. In addition, antibacterial mechanisms triggered by loperamide induced a significant reduction in bacterial load and an upregulation of BPI and LL37 gene expression. Thus, our results show that loperamide exerts immunomodulatory effects, which supports its use for additional medical conditions other than diarrhea.

1. Introduction

Previous studies demonstrated that induction of autophagy by drugs such as loperamide could improve the bactericidal activity of host cells and may be useful in tuberculosis treatment as an adjunctive therapy [1]. Loperamide is a phenylpiperidine derivative commonly used to treat infectious and noninfectious, acute and chronic diarrhea [2,3]. Loperamide targets two types of membrane receptors, voltage-dependent and voltage-independent calcium channels and μ- and δ-opioid receptors [4–7]. As loperamide can mediate intracellular calcium levels by blocking calcium channels and activating opioid receptors, several biological effects of loperamide have been described in addition to those involved in diarrhea control, suggesting its potential uses for alleviating pain, controlling anxiety, reducing insulin resistance and inhibiting coronavirus replication [4,8–10]. The advantages of performing research dedicated to repurposing currently available drugs include a considerable reduction in the time and costs required to discover new therapeutic approaches [11].

We recently observed that loperamide enhances control of a *M. tuberculosis* strain in human and murine alveolar macrophages by inducing autophagy. Furthermore, this antimicrobial activity is associated with a reduction of TNFα production, suggesting that loperamide may play a role in preventing excessive inflammation [1]. The regulation of proinflammatory cytokine production is desirable during *M. tuberculosis* infection, because cytokines such as TNFα and type I and II interferons, despite their protective roles in immunity against *M. tuberculosis*, can induce a hyperinflammatory state associated with tissue damage and tuberculosis pathogenesis [12,13]. Loperamide may induce additional immunoresponses associated with its effect on calcium channels and opioid receptors. The blockade of intracellular calcium influx has been

Abbreviations: BPI, bactericidal/permeability increasing protein; LL37, cathelicidin LL37; FDA, Food and Drug Administration; PBMC, peripheral blood mononuclear cells; BCG, Bacillus Calmette-Guérin; CFUs, Colony forming units; MOI, multiplicity of infection; GM-CSF, granulocyte-monocyte colony stimulating factor; M1, classically activated macrophages; M2, alternatively activated macrophages

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shown to cause a reduction in the production of the LPS-dependent proinflammatory cytokines IL6, TNFα, MCP-1 and IFNγ in murine macrophages [14]. Activation of μ-opioid receptors induces the production of the proinflammatory chemokines TNFα and prostaglandin E2 (PGE2) in endothelial cells and human peripheral blood mononuclear cells (PBMCs) [15,16]. Thus, loperamide may have an immunomodulatory activity that could be useful as an adjunctive therapy against *M. tuberculosis* infection and should be evaluated to elucidate its utility in new clinical indications.

In this study, our goal was to assess the ability of loperamide to regulate human macrophage responses to infection with the virulent strain of *M. tuberculosis*. We measured the proinflammatory mediator profile and the induction of two antimicrobial peptides, bactericidal/permeability increasing protein (BPI) and cathelicidin LL37. Both BPI and LL37 may be involved in inducing *M. tuberculosis* death, and both are known to downregulate proinflammatory mediator production in addition to their antimicrobial peptide functions. We observed that loperamide exerted an immunomodulatory effect on TNFα and antimicrobial peptide production by human macrophages infected with *M. tuberculosis* and that these responses were independent of the bacteria, as they also occurred when macrophages were infected with *M. smegmatis* and to a lesser extent with *M. bovis*. In addition, the antibacterial mechanisms triggered by loperamide included upregulation of BPI and LL37 gene expression via μ-opioid receptor-dependent immunomodulatory responses. Although there are limitations to the extent to which human diseases are fully represented by in vitro models, such models have the potential to contribute to the early identification and prioritization of active molecules that induce signals relevant to disease biology in studies that aim to repurpose existing drugs.

2. Methods

2.1. Cells

Monocytes were isolated from PBMCs by positive selection using magnetic beads (Miltenyi Biotech, Auburn, CA) from buffy coats of samples from healthy blood bank donors at the Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas. Cell suspension purity and viability were assessed by flow cytometry, revealing that 97.38 ± 1.26% of cells were CD14+ and cell viability was 99.4 ± 0.2% (mean ± SE). The monocyte suspension was adjusted to 1×10^6 cells/mL in RPMI-1640 supplemented with 200 mM L-glutamine (Lonza, Walkersville, MD), 5 μg/L gentamicin sulfate (Lonza) and 10% heat-inactivated human serum (Valley Biomedicals, Winchester, VA). We cultured human monocytes for 7 days to generate monocyte-derived macrophages (macrophages). At this time, a cell viability of 95 ± 2.5% was observed, as assessed by trypan blue exclusion. These studies were approved by the Institutional Review Board of the Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas. The design of the subsequent in vitro studies followed the recommendations of Curtis and colleagues [17].

2.2. Mycobacteria preparation

Mycobacterium tuberculosis H37Rv (*M. tuberculosis*), *M. bovis* BCG and *M. smegmatis* (ATCC, Manassas, VA) were cultured in Middlebrook 7H9 broth medium (Difco Laboratories, Detroit, MI). After incubating *M. tuberculosis* and *M. bovis* BCG for 21 days and *M. smegmatis* for 3 days at 37 °C, the mycobacterial stock solution was harvested, aliquoted and stored at −80 °C until use. Colony-forming units (CFUs) were enumerated after the disruption of mycobacterial clumps [18].

2.3. Infection with mycobacteria and loperamide treatment

Macrophages (2×10^5 macrophages/well in 96-well polystyrene plates) were infected with *M. tuberculosis*, *M. bovis* BCG or *M. smegmatis* in RPMI with 30% nonheat-inactivated pooled human AB serum and without gentamicin at an MOI of 5 (5:1, bacteria:macrophage). The cells were then incubated for 1 h followed by three washes to remove any nonphagocytosed bacteria. Macrophages were then cultured for another hour in RPMI supplemented with 10% heat-inactivated pooled human serum with or without 3 μM loperamide, a concentration that was determined in pilot assays [1]. The cells were cultured for 24 h before collecting the supernatants and cell lysates, which were stored at −20 °C until use. The supernatants were centrifuged at 340 × *g* using Spin-X tubes with a 0.2 mm membrane (Corning, New York) to remove bacteria, macrophages and cell debris. For comparison purposes, we included uninfected cells treated only with loperamide, LPS or vehicle as controls.

2.4. Antimicrobial activity

To evaluate the effects of loperamide on mycobacterial intracellular growth control, 2×10^5 macrophages/well in 96-well polystyrene plates were infected with bacteria at an MOI of 5 and treated with loperamide after extensive washing to remove nonphagocytosed bacteria. Macrophages infected with *M. tuberculosis* were further incubated at 37 °C in a 5% CO_2 atmosphere for 1 h (Day 0) and 72 h (Day 3). *M. smegmatis*-infected macrophages were cultured for 24 h after the loperamide treatment, with a set of control experiments performed in which cells were detached and counted to determine the number of recovered macrophages and to assess macrophage viability via a trypan blue exclusion assay. After the *M. smegmatis*-infected cell supernatants were discarded, the cells were lysed with 0.1% SDS for 10 min and then neutralized with 20% BSA. The lysates were serially diluted and plated onto 7H10 agar plates in triplicate. The CFUs of *M. tuberculosis* were enumerated after 21 days, and the CFUs of *M. smegmatis* were enumerated after 3 days of growth at 37 °C. In selected experiments, 1 μM naltrexone was added 30 min before the loperamide treatment.

2.5. Cytokine production

Supernatants from 24 h cultures were assayed for the release of IL1β, IL6, IL8, IL10, IL12p70 and GM-CSF using Bio-Plex human cytokine customized detection kits (Bio-Rad, Hercules, CA, US) following the manufacturer's protocol. In selected experiments, TNFα and IL10 levels were measured using an in-house ELISA with specific pairs of antibodies, including anti-human TNFα (Pharmingen, San Diego, CA) or anti-IL10 (Probiotek, Monterrey, MX), as previously described [18]. Supplemented RPMI-1640 was used as a negative control. Absorbance was read using a Multiskan Ascent Microplate Reader (Thermo Fisher Scientific, Waltham, MA) at 405 nm. The results are presented as the mean value of duplicate wells.

2.6. Reverse transcription and real-time PCR

Total RNA was extracted and reverse transcribed. Subsequently, the cDNA was used in quantitative real-time PCR (qRT-PCR, TaqMan) assays to determine mRNA expression levels using the comparative threshold cycle method (ΔΔCt), as previously described [19]. Real-time PCR reactions were performed in duplicate wells according to the manufacturer's protocol for TaqMan predesigned gene assays. The TNFα (Hs00174128_n1), IL1β (Hs01555410_n1), IL10 (Hs00961622_n1), COX-2 (Hs000153133_m1), LL37 (Hs00189038_m1) and BPI (Hs01552756_m1) genes were evaluated, and all gene assays were purchased from Applied Biosystems (Carlsbad, CA). The Ct values for each gene were normalized to the endogenous 18S rRNA control gene (4319413E), where the threshold number of reference molecules and initial number of reference molecules is taken into account, eliminating the need for quantifying the initial amounts of cDNA or cell numbers. With this method, the amount of target DNA, normalized to an endogenous reference, is calculated relative to a calibrator

(unstimulated or any selected condition), provided that the efficiencies of the target and reference amplifications are validated (Applied Biosystems user Bulletin #2).

2.7. Loperamide signaling inhibition

To assess ligand-induced responses, macrophages were stimulated using 3 μM loperamide for 24 h. To block loperamide signaling, 0.1, 1 or 10 μM naltrexone or Bay K8644 was added to the cells 30 min before loperamide stimulation. Next, the supernatants were collected and then frozen until cytokine assessment. The cells were harvested and lysed for mRNA extraction. Culture medium alone was used as a negative control, and cells treated with LPS were used as a positive control, as indicated.

2.8. Data and statistical analysis

We used Friedman's ANOVA followed by Dunn's posttest to assess differences among treatments. Statistical analyses were performed with Prism version 6.0 for Mac (GraphPad Software, San Diego, CA), and $p < 0.05$ was considered significant.

2.9. Materials

Loperamide, Bay K8644 (B112), and naltrexone were purchased from Sigma Aldrich (St. Louis, MO). Reagents were dissolved either in dimethyl sulfoxide (DMSO, Sigma Aldrich) or ethanol (Baker). Culture proportions of DMSO or ethanol were lower than 0.1%. The final culture concentrations of all reagents used in the reported assays were determined in pilot assays. Macrophage viability was assessed using the Cell Titer cell viability assay (Promega, Madison, WI, USA), and no significant reduction in viability was observed using the selected concentrations (Supplementary Fig. 1a–c).

3. Results

3.1. Loperamide induces the killing of M. tuberculosis in macrophages

Our previous report showed that loperamide enhanced alveolar macrophage antibacterial responses against *M. tuberculosis* H37Rv [1]. To model any additional immunomodulatory effects of loperamide, we first demonstrated that the same antibacterial response occurred in monocyte-derived macrophages. We observed a significant reduction in intracellular CFUs when the macrophages were treated with loperamide (Fig. 1a). Furthermore, loperamide upregulated expression of the genes encoding antimicrobial peptide BPI (Fig. 1b) and cathelicidin LL37 (Fig. 1c).

3.2. Loperamide modulates the proinflammatory environment associated with M. tuberculosis infection

We next evaluated the ability of loperamide to modulate the inflammatory environment in the context of *M. tuberculosis* infection. We showed that the reduced bacterial load observed in infected macrophages treated with loperamide was accompanied by an increase in IL1β production at both the protein and transcriptional levels. We observed a significant increase in IL10 and a significant reduction in TNFα production at both the protein and transcriptional levels. In addition, we observed increases in PGE2 production and COX-2 gene expression, the enzyme responsible for PGE2 synthesis, in infected macrophages treated with loperamide. The addition of LPS to infected macrophages induced a significant increase in cytokine production compared to that in nontreated cells (Fig. 2). The production of significant amounts of IL6 and GM-CSF were induced in infected macrophages compared to nontreated cells (medium) (Supplementary Fig. 2). Changes in the production of IL12p70 were not significant in any of the treatments assessed. In addition, the production of IL8 was not modified under loperamide or LPS treatments (Supplementary Fig. 2). Control experiments demonstrated that loperamide alone did not affect the macrophage numbers nor their viability (100% of macrophages remained in the wells and they were 96.3 ± 3.1% viable) over the course of the culture time. Bacterial infection reduced the number of cells and their viability (75 ± 10% of remaining cells were 80 ± 13% viable). The addition of loperamide slightly prevented cell loss (81.6 ± 13% of remaining cells were 98 ± 1.3% viable) (Supplementary Fig. 1a and d).

3.3. Regulation of inflammation following infection with other mycobacteria

As the observed regulation of inflammation may have been a general effect of the induction of IL10 production by loperamide that was independent of *M. tuberculosis* infection, we further evaluated TNFα and IL10 production upon treatment with loperamide alone. We observed that loperamide did not induce the production of TNFα but did induce the production of significant amounts of IL10 in macrophages (Fig. 3a). Furthermore, the reduction in TNFα production and CFU counts was not an effect of the ethanol used as vehicle (Fig. 3b). To assess whether the regulatory effect of loperamide on TNFα production was dependent on *M. tuberculosis* infection or on proinflammatory responses to other stimuli, we treated macrophages with loperamide and stimulated them with LPS or infected with *M. bovis* BCG or *M. smegmatis*. We observed that loperamide induced a decrease in TNFα production regardless of the inducer, but the observed reduction was only significant when cells were infected with *M. smegmatis* (Fig. 3c). Because infection with *M. smegmatis* provided a good model to further characterize the biological effects of loperamide, and because it allowed experiments to be performed at a lower laboratory biosafety level, we used this

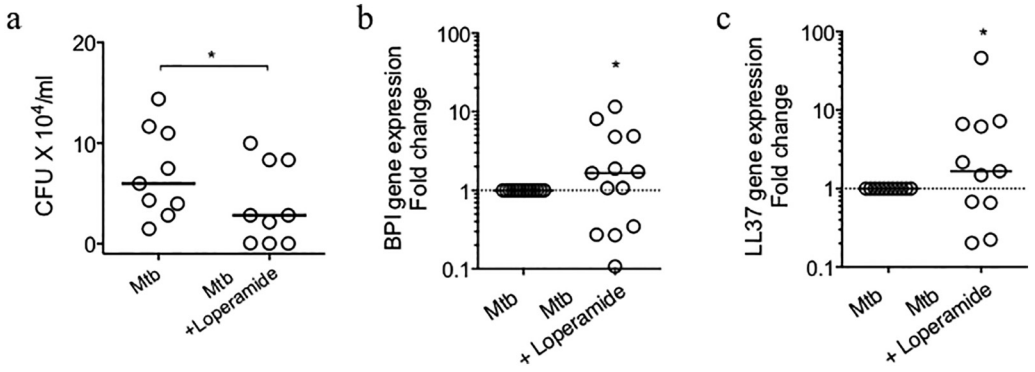

Fig. 1. Loperamide induces antimicrobial responses in human macrophages infected with *M. tuberculosis*. Macrophages were infected with *M. tuberculosis* H37Rv at an MOI of 5 for 1 h. After extensive washing, the cells were treated with 3 μM loperamide for an additional 3 days (a) or 24 h (b and c). The intracellular bacterial loads were evaluated by counting the CFUs (a) ($n = 9$, $^*p < 0.05$). Cell lysates were obtained for quantitative PCR to assess BPI protein ($n = 13$) and cathelicidin LL37 ($n = 11$) levels relative to those of unstimulated cells, with experiments performed in duplicate. The individual values are depicted. $^*p < 0.05$ vs control.

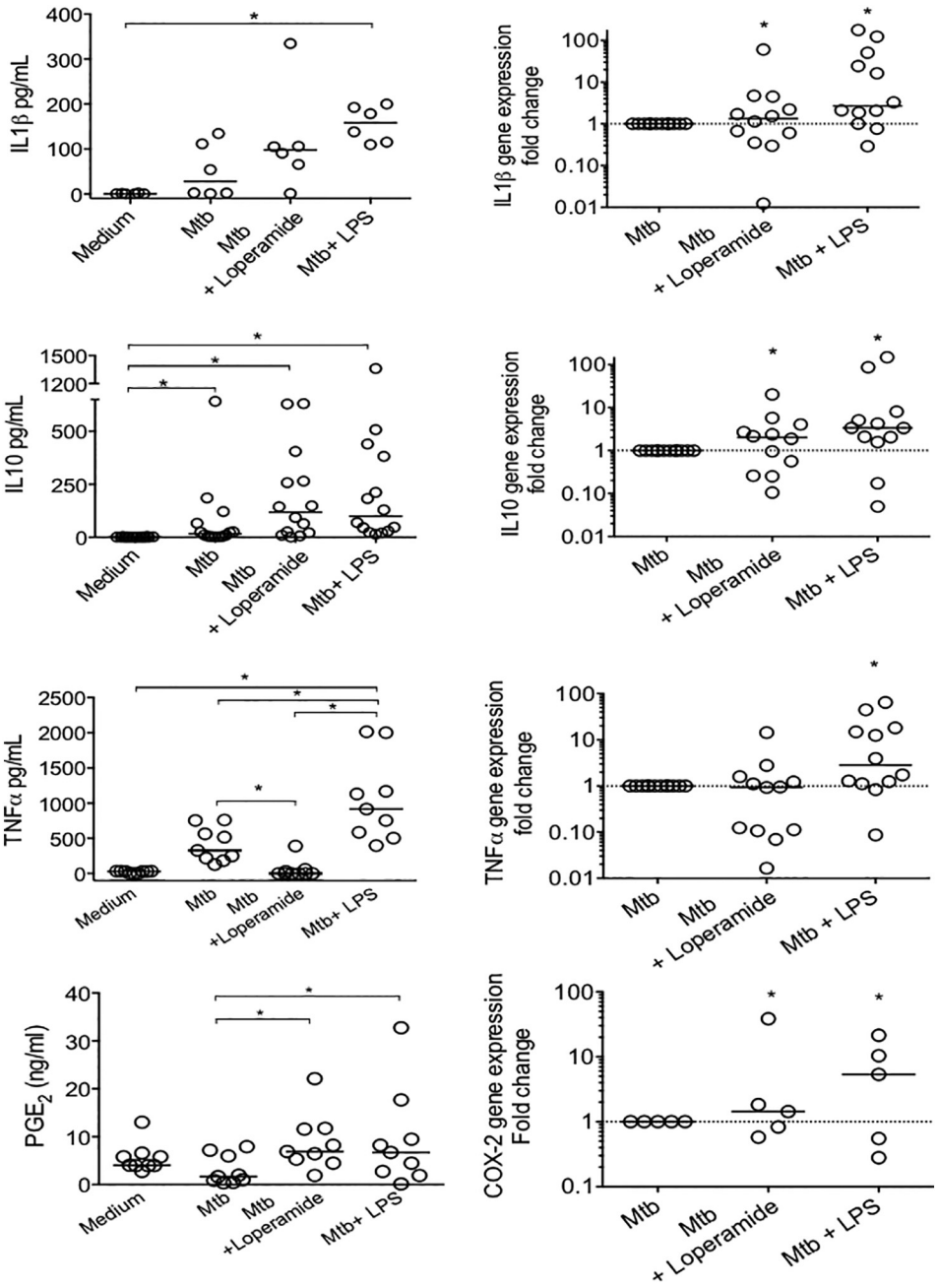

Fig. 2. Loperamide modulates the proinflammatory environment of infected macrophages. Macrophages were infected with *M. tuberculosis* H37Rv at an MOI of 5 for 1 h. After extensive washing, the cells were treated with 3 μM loperamide for an additional 24 h. The production of IL1β, IL10, and TNFα was measured in the culture supernatants using a customized Luminex assay, and PGE2 was measured by ELISA (left). The individual values are depicted; *$p < 0.05$, Friedman's ANOVA followed by Dunn's multiple comparison test. Cell lysates were obtained to assess IL1β, IL10, TNFα and COX-2 gene expression in infected cells treated with loperamide relative to that in untreated infected cells by quantitative PCR, with experiments performed in duplicate. The individual values are depicted. *$p < 0.05$ vs control.

mycobacterium in subsequent experiments.

3.4. Loperamide regulates immune responses through specific receptors

We next sought to determine whether the effects of loperamide on macrophages depended on its known receptor-activating function. Because loperamide blocks calcium channels, we used Bay K8644 (B112), a calcium ionophore, to increase intracellular calcium levels before treatment with loperamide. Additionally, because loperamide is an activator of μ-opioid receptors, we used naltrexone, a generic inhibitor of opioid receptors (mu, delta, kappa and sigma) prior to treating *M. smegmatis*-infected macrophages with loperamide.

We observed that neither Bay K8644 (Fig. 4a) nor naltrexone (Fig. 4b) restored TNFα production to levels observed in *M. smegmatis*-infected macrophages alone. However, compared to the medium control, the production of TNFα was significantly augmented in macrophages to which 1 and 10 μM naltrexone was added to prevent activation of μ-opioid receptors, whereas TNFα levels did not significantly change in *M. smegmatis*-infected cells treated with loperamide. Responses observed in the presence of Bay K8644 were more variable, and at 1 μM Bay K8644, the levels of TNFα were significantly higher than those of the background.

To further determine whether calcium channel or opioid receptor activation by loperamide induces antimicrobial activity, we treated infected macrophages with Bay K8644 and naltrexone prior to loperamide stimulation and enumerated the intracellular bacteria. We observed significantly reduced numbers of intracellular bacteria in macrophages treated with loperamide (Fig. 5a) and a concomitant reduction in TNFα production (Fig. 5b). The inhibition of opioid receptor signaling with naltrexone partially neutralized the effect of loperamide on the antimicrobial activity and restored the production of TNFα. The augmentation of intracellular calcium influx did not neutralize the effects of loperamide on microbial killing or TNFα production. Furthermore, neither ethanol (EtOH, vehicle of loperamide) nor

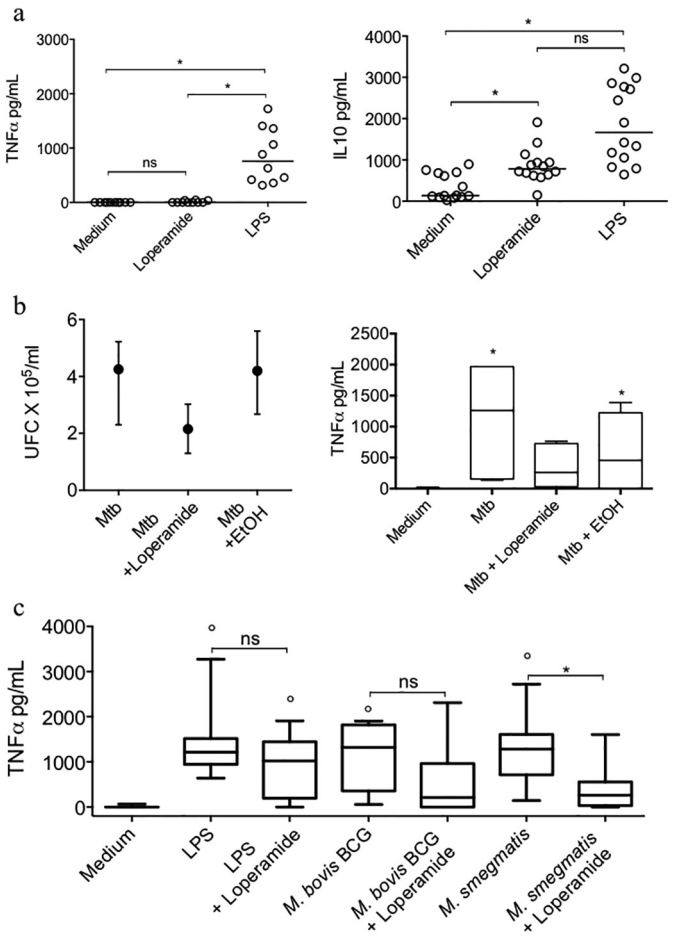

Fig. 3. Loperamide downregulates TNFα production in mycobacterium-infected macrophages. (a) Cells were stimulated with 3 μM loperamide or 100 ng/mL LPS for 24 h. TNFα (n = 10) and IL10 (n = 14) production was determined in the culture supernatants by ELISA. The individual results are depicted, and lines indicate the medians; *p < 0.05, ns = not significant. (b) Cells were infected with *M. tuberculosis* H37Rv at an MOI of 5 for 1 h. After extensive washing, the cells were treated with 3 μM loperamide for an additional 3 days (left) or 24 h (right). The intracellular bacterial loads were evaluated by counting the CFUs, and the levels of TNFα in the culture supernatants were determined by ELISA. CFUs are depicted as medians with interquartile range and TNFα as boxplots indicating quartiles and medians, n = 6, *p < 0.05 vs medium. EtOH, ethyl alcohol, used as control. (c) Cells were treated with 100 ng mL^{-1} LPS or infected with *M. bovis* BGC or *M. smegmatis* at an MOI of 10 for 1 h. After extensive washing to discard nonphagocytized bacteria, the cells were treated with 3 μM loperamide for additional 24 h. TNFα production was determined in the culture supernatants by ELISA. Box plots indicate quartiles and medians, n = 11. °p < 0.05 vs medium, *p < 0.05 between selected pairs, ns = not significant, Friedman's ANOVA followed by Dunn's multiple comparison test.

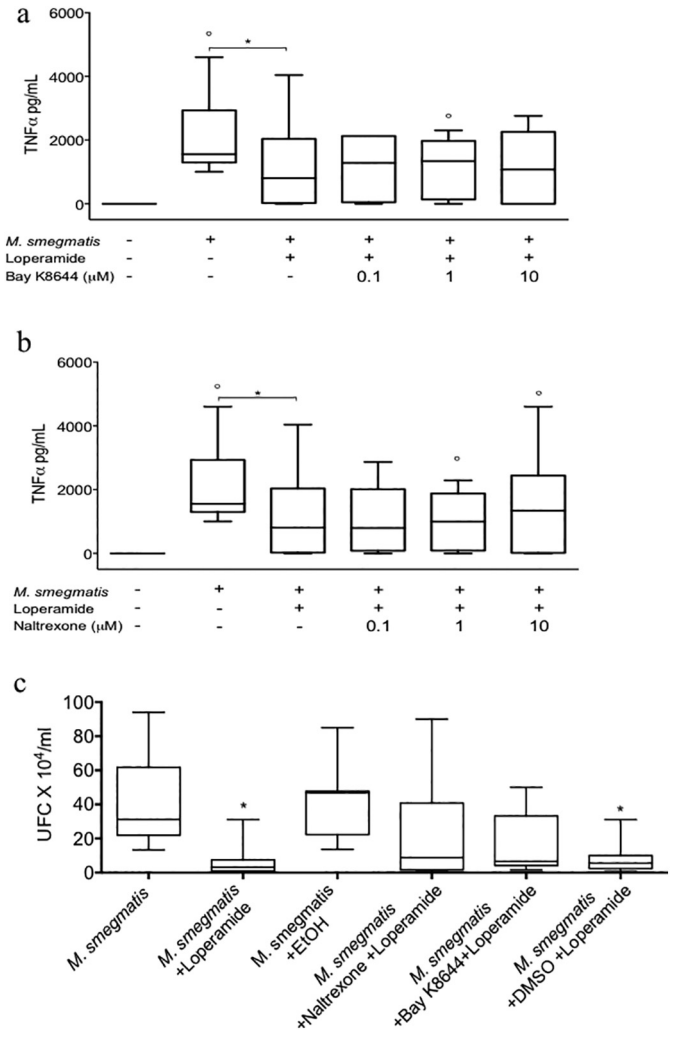

Fig. 4. Effect of increased intracellular calcium influx or opioid receptor blockade on immunomodulation by loperamide. Macrophages were infected with *M. smegmatis* at an MOI of 10 for 1 h. After extensive washing to discard nonphagocytized bacteria, cells were treated with increasing concentrations of (a) Bay K8647 or (b) naltrexone for 30 min followed by treatment with 3 μM loperamide for an additional 24 h. TNFα production was in the culture supernatants was assessed by ELISA. Box plots indicate quartiles and medians, n = 10. °p < 0.05 vs medium, *p < 0.05 between selected pairs, Friedman's ANOVA followed by Dunn's multiple comparison test.

DMSO (vehicle of Bay K8644 and naltrexone) modified TNFα production or the antimicrobial activity of infected macrophages. Because we observed a partial contribution of opioid receptors to the antimicrobial activity induction, we further evaluated the effect of naltrexone on the expression of BPI and LL37 in macrophages infected with *M. smegmatis* (Fig. 6a and b) or *M. tuberculosis* (Fig. 6c and d). We observed that loperamide-dependent upregulation of BPI and LL37 gene expression in macrophages infected with *M. tuberculosis* was neutralized by the addition of naltrexone (Fig. 6c and d).

4. Discussion

The development of novel pharmaceuticals can be deterred by the need to characterize drug pharmacokinetics, dosing, and safety information in humans [11]. Developing a new drug from a promising molecule with the aim of bring it to market can take ten years or more, and the cost is considerably high [20]. Consequently, translational research to bring laboratory findings to the bedside of patients with tuberculosis is challenging. Therefore, a key strategy is repurposing currently available drugs that have potential uses in treating tuberculosis. Our previous report showed that loperamide is able to stimulate human and murine macrophages infected with a virulent *M. tuberculosis* strain to elicit antimicrobial responses by inducing autophagy [1]. In this study, we evaluated the immunomodulatory activity of loperamide in the context of a mycobacterial infection.

We observed that loperamide increased the bactericidal activity and induced the overexpression of the antimicrobial peptides BPI and LL37 in macrophages infected with *M. tuberculosis*. The antimycobacterial activity of BPI and LL37 has been reported by others [21,22], and the induction of antimicrobial peptides is relevant in tuberculosis. In addition, it has been reported that the expression of both peptides is decreased in tuberculosis [23]. To the best of our knowledge, this is the

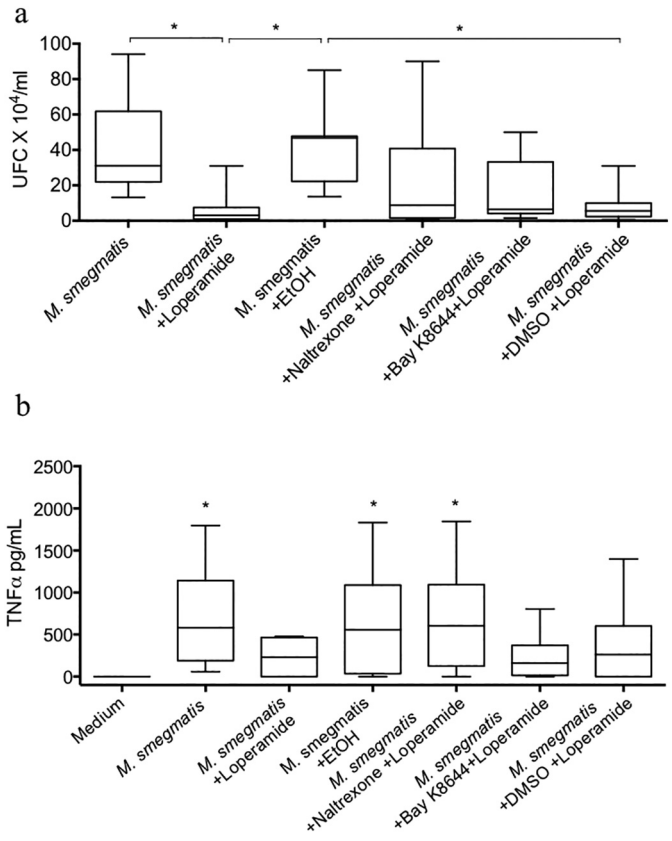

Fig. 5. Effect of increased intracellular calcium influx or opioid receptor blockade on the antimicrobial activity induced by loperamide. Macrophages were infected with *M. smegmatis* at an MOI of 10 for 1 h. After extensive washing to remove nonphagocytized bacteria, the cells were treated with 1 μM Bay K8647 or naltrexone for 30 min followed by treatment with 3 μM loperamide for an additional 24 h. The intracellular bacterial loads were evaluated by counting the CFUs (a); n = 8, *$p < 0.05$ between selected pairs. TNFα production was determined in the culture supernatants by ELISA (b). EtOH and DMSO were used as controls. Box plots indicate quartiles and medians, n = 8, *$p < 0.05$ vs medium, Friedman's ANOVA followed by Dunn's multiple comparison test.

first study to report that loperamide is associated with the induction of the antimicrobial peptides BPI and LL37 in human cells, although the mechanisms associated with loperamide-dependent induction of BPI and LL37 production are unknown. In macrophages, the best studied inducer of LL37 is vitamin D, and the induction of LL37 by vitamin D is dependent on the synthesis of the active form of 1,25(OH)(2)D and the enzyme 25-hydroxyvitamin D-1alpha-hydroxylase (CYP27B1) [22]. BPI is induced in human macrophages by several PAMPs, such as LPS and flagellin, during bacterial infection [24]. The mechanism involved in BPI and LL37 induction by loperamide may include distinct signal transduction pathways that converge on a common transduction signal of both antimicrobial peptides. Thus, we demonstrated that in addition to the antimicrobial activity associated with autophagy induction [1], loperamide treatment induces antimicrobial activity in macrophages infected with *M. tuberculosis* and *M. smegmatis* in association with antimicrobial peptide production.

We also observed that loperamide treatment induced a reduction in TNFα levels and increased the production of IL10 and PGE2 in macrophages infected with *M. tuberculosis*. The role of loperamide in regulating inflammation during infection has not been previously reported, and we observed that it was not restricted to *M. tuberculosis* infections, as this activity as also observed in cells infected by the mycobacteria *M. smegmatis* and *M. bovis*. Because IL10 exerts a suppressive effect on TNFα production [25], the induction of IL10 by loperamide may explain the reduction in TNFα production. IL-1β has been reported to partially act through COX2 to induce PGE2 synthesis, which regulates the containment of *M. tuberculosis* [26]. Therefore, the induction of IL1β production by loperamide may participate in mycobacterial killing through the incremental increase in COX-2 and PGE2 levels. In addition, the antimicrobial peptides BPI and LL37 can both modulate host immune responses and participate in the regulation of cytokine expression by targeting inflammatory pathways such as Toll-like receptor and NF-kB [27–29], suggesting that the induction of BPI and LL37 production by loperamide also plays a role in the immunoregulation of TNFα, IL10 and PGE2 production. Under induced stress conditions, murine macrophages have been observed to display an intermediary macrophage phenotype between M1, characterized by high antibacterial activity (with LL37 and extracellular trap induction) and M2, characterized by increased IL-10 production and reduced TNFα production [30]. We observed that loperamide induced the production of high levels of IL1β, IL6, GM-CSF, BPI and LL37, which is associated with an M1 phenotype, and high levels of IL10 and PGE2, which is associated with an M2 phenotype. This loperamide-induced cytokine and antimicrobial peptide profile resembles an intermediary macrophage phenotype [30].

We further investigated the signaling pathways responsible for loperamide modulation of TNFα production using the *M. smegmatis* infection model, as it induced higher amounts of TNFα than *M. tuberculosis* and allowed for better discrimination of the roles of various pathways, with the additional advantage of it being a fast-growth mycobacterium. Pretreatment of macrophages with a calcium ionophore (Bay K8644) did not restore the inhibition of *M. smegmatis*-induced TNFα production by loperamide, suggesting that the blockade of calcium channels by loperamide was unlikely to be responsible for the observed modulation of TNFα production. This phenomenon was unexpected, because *M. smegmatis* induced-production of TNFα largely depends on Ca^{2+} signaling [31]. However, the role of calcium in mediating TNFα production is controversial, because increased intracellular Ca^{2+} due to calcium influx (channel-mediated) exerts an inhibitory effect on LPS-induced TNFα production [32]. In contrast, the inhibition of calcium influx also causes reduced TNFα production and decreased protection against excess inflammation [33,34]. A negligible participation of the calcium blockade on the TNFα production and antimicrobial responses induced by loperamide was observed in mycobacterium-infected macrophages, as the restoration of intracellular calcium levels by Bay K8644 did not prevent *M. smegmatis* killing. This phenomenon was unexpected, because calcium channel blockade triggers antimicrobial autophagy [35,36]. *M. tuberculosis* has been reported to interfere with macrophage microbicidal mechanisms and to inhibit murine and human macrophage Ca^{2+} signaling to evade immune detection or decrease phagolysosome formation and macrophage reactive oxygen species production, contributing to the intracellular survival of *M. tuberculosis* [37]. Thus, loperamide is likely important in inducing other bactericidal mechanisms that are unrelated to the blockade of Ca^{2+} influx.

As loperamide is a potent μ-opioid receptor agonist, we explored whether the immunomodulation induced in loperamide-treated macrophages was related to activation of μ-opioid receptors. We evaluated the effect of naltrexone, an opioid antagonist that effectively neutralizes loperamide gastric pathways [38], observing that naltrexone restored *M. smegmatis*-induced TNFα production in loperamide-treated macrophages. This finding suggests that the activation of opioid receptors by loperamide was, at least partially, involved in the regulation of TNFα production. In addition, we observed that activation of opioid receptors by loperamide also mediated the antimicrobial activity of macrophages, as naltrexone partially abrogated the loperamide-dependent bacterial clearance and completely abrogated the upregulated BPI and LL37 gene expression, which was not observed upon treatment with the calcium ionophore. A relationship between opioid receptors and antimicrobial peptides has not previously been reported. Other possible explanations

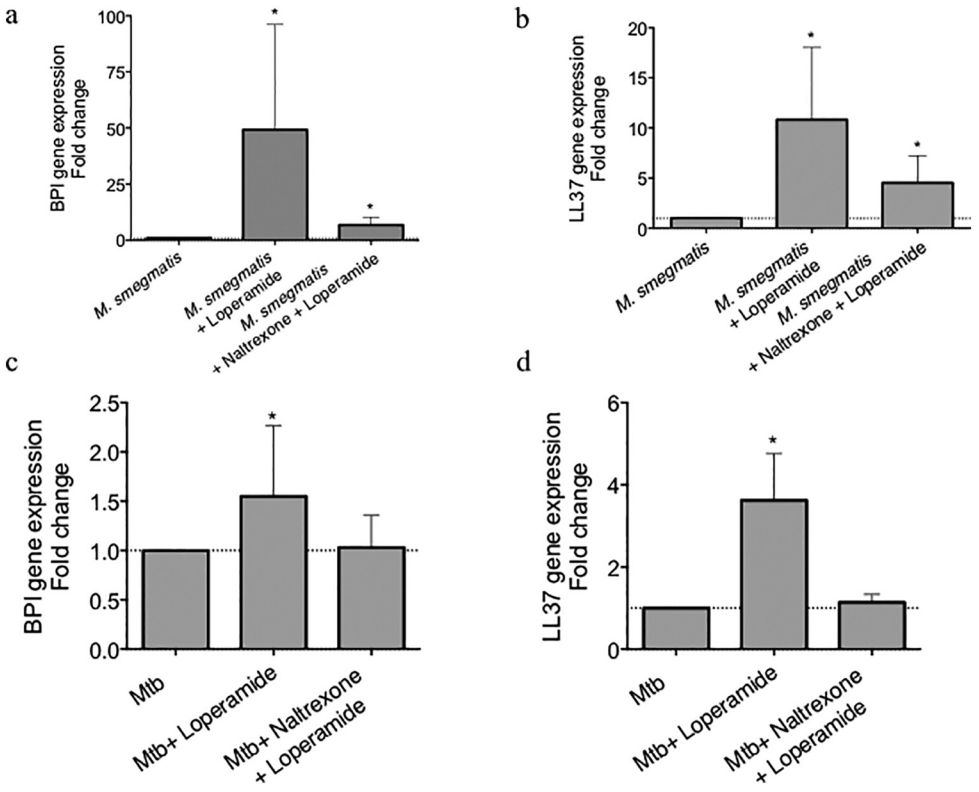

Fig. 6. Loperamide-dependent BPI and LL37 gene expression are regulated by μ-opioid receptor signaling. Macrophages were infected with *M. smegmatis* (a, b) or *M. tuberculosis* (c, d) at an MOI of 5 for 1 h. After extensive washing to discard nonphagocytized bacteria, the cells were treated with 1 μM naltrexone for 30 min followed by treatment with 3 μM loperamide for an additional 24 h. Cell lysates were obtained to assess BPI and LL37 gene expression relative to that of untreated mycobacterium-infected cells by quantitative PCR, with experiments performed in duplicate. The means ± SEM of 6 independent experiments are depicted. *$p < 0.05$ vs control.

for loperamide activation of innate responses in *M. tuberculosis*-macrophages were beyond the scope of this study, and future studies will be needed to ascertain their integrative function and relative priority.

In conclusion, loperamide induces μ-opioid receptor-dependent immunomodulatory responses and bactericidal mechanisms that include the induction of antimicrobial peptides and the reduction of TNFα production in human macrophages infected with *M. tuberculosis* or *M. smegmatis*. Thus, loperamide stimulation increases the ability of macrophages to fight bacterial infections while protecting tissues from damage caused by excessive inflammation. These results support the potential use of loperamide for treating infectious diseases such as mycobacterial infections. In addition, our findings suggest that agonist-dependent μ-opioid receptor signaling in human macrophages has potential roles in the immunomodulation of infectious diseases. A better understanding of the agonist-specific immunomodulatory effects of opioids will offer additional possibilities for selecting optimal drugs for therapies that confer more favorable and/or less detrimental side effects to immune cells.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Author contributions

E.J.: conducted the experiments, analyzed the data and prepared the manuscript; A.R.: conducted the experiments; O.H.: conducted the experiments; E.S.: designed the experiments and prepared the manuscript; M.T.: designed the experiments, analyzed the data and prepared the manuscript.

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