International Immunopharmacology 74 (2019) 105694

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

# Resolvin D1 (RvD1) and maresin 1 (Mar1) contribute to human macrophage control of *M. tuberculosis* infection while resolving inflammation

Andy Ruiz[a,b], Carmen Sarabia[a], Martha Torres[a], Esmeralda Juárez[a,*]

[a] Departamento de Investigación en Microbiología, Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas, CDMX 14080, Mexico
[b] Posgrado en Ciencias Biológicas, Facultad de Medicina, Universidad Nacional Autónoma de México, CDMX 04510, Mexico

ARTICLE INFO

*Keywords:*
Resolution of inflammation
Resolvin D1
Maresin 1
Tuberculosis
Macrophages

ABSTRACT

Resolvins and protectins counter inflammation, enhance phagocytosis, induce bactericidal/permeability-increasing protein (BPI) expression, and restore inflamed tissue to homeostasis. Because modulating the inflammation/antiinflammation balance is important in *Mycobacterium tuberculosis* infection, we evaluated the effects of resolvins and protectins on human macrophages infected *in vitro*. Monocyte-derived macrophages were infected with *M. tuberculosis* H37Rv at a multiplicity of infection (MOI) of 5 and treated 1 h post-infection *in vitro* with 100 nM LXA4, RvD1, RvD2, PD1 or 150 nM Mar1. After 24 h, cytokine production was measured by Luminex, and BPI and cathelicidin LL37 expression was determined by real-time PCR. Macrophage bactericidal activity was assessed by colony-forming units (CFUs) 3 days posttreatment. Nuclear translocation of Nrf2 was assessed by ELISA, NFκB translocation was determined by imaging cytometry, and BPI production was determined by fluorescence microscopy. We found that all lipids reduced LPS-dependent and *M. tuberculosis*-induced TNF-α production. RvD1 and Mar1 also induced a significant reduction in *M. tuberculosis* intracellular growth. RvD1 and Mar1 elicited distinct immunomodulatory patterns. RvD1 induced upregulation of both antimicrobial effector genes (BPI and LL37) and cytokines (GM-CSF and IL-6). Mar1 induced only BPI overexpression. RvD1 and Mar1 induced NFκB nuclear translocation, but only Mar1 induced Nrf2 translocation. Inhibition of G protein-coupled receptor signaling in infected macrophages abrogated the regulatory effects of RvD1. In conclusion, RvD1 and Mar1 modulate the anti-inflammatory and antimicrobial properties of *M. tuberculosis*-infected human macrophages. Since both proresolving lipids are inducible and synthesized from dietary components, they have immunotherapeutic potential against tuberculosis when inflammation is uncontrolled.

## 1. Introduction

*M. tuberculosis*, the causative agent of tuberculosis, is the leading bacterial cause of human mortality worldwide. In 2017, tuberculosis caused an estimated 1.3 million deaths (range, 1.2–1.4 million) [1]. *M. tuberculosis* enters the lung and penetrates the alveolar space, where the bacteria are taken up by alveolar macrophages, which respond to infection by producing proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α. Proinflammatory cytokines recruit other immune cells to the lung and activate antimicrobial pathways in macrophages to control the infection. However, if the inflammatory environment persists, macrophages become permissive for *M. tuberculosis* intracellular growth, preventing bacterial clearance [2], and severe histopathological damage occurs in the lungs that promotes the transmission of the disease [3–5]. In our previous studies, we found that control of *M. tuberculosis* infection occurs in low-inflammation environments [6–8], which suggests that regulators of inflammation participate at different levels in antimicrobial responses.

The resolution of inflammation, also known as catabasis, is a process that returns the host tissues to a noninflammatory state [9]. During the resolution of inflammation, there is a reduction in neutrophil influx and an increase in neutrophil apoptosis; the macrophages clear the inflammatory milieu by phagocytosis of microbes and apoptotic neutrophils [10]. In addition, as tissue leukocytes recede, the levels of proinflammatory cytokines and chemokines also decrease, and tissue repair mechanisms are activated to complete the return to homeostasis. The resolution process depends critically on the production of oxygenated lipids derived from essential polyunsaturated fatty acids (PUFAs)

* Corresponding author at: Departamento de Investigación en Microbiología, Instituto Nacional de Enfermedades Respiratorias, Calzada de Tlalpan 4502, Sección XVI, México City 1408, Mexico.
*E-mail address:* ejuarez@iner.gob.mx (E. Juárez).

https://doi.org/10.1016/j.intimp.2019.105694
Received 16 March 2019; Received in revised form 26 May 2019; Accepted 10 June 2019
Available online 19 June 2019

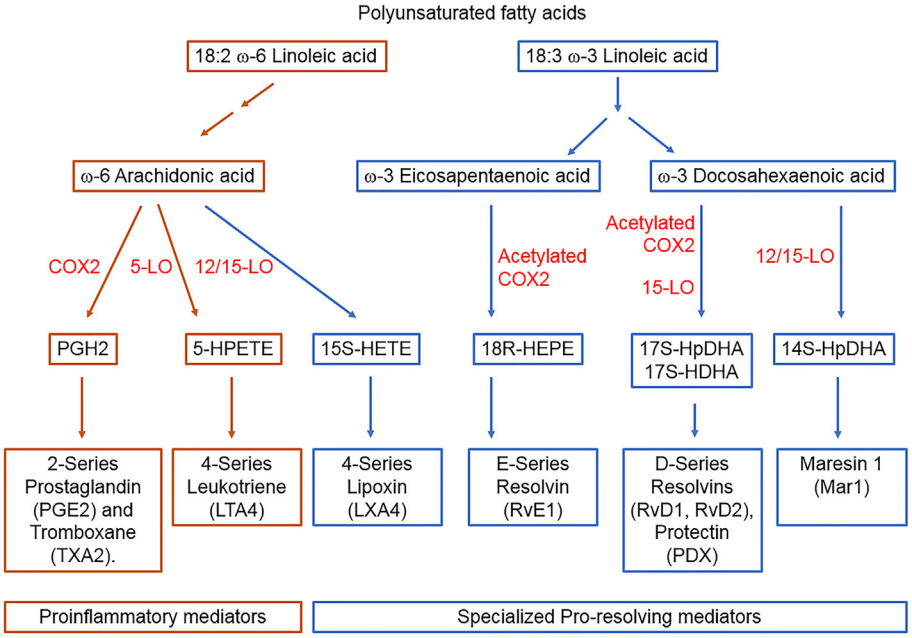


**Fig. 1.** Generalized pathway for the conversion of polyunsaturated fatty acids to proinflammatory and proresolving mediators. COX, cyclooxygenase; LO, lipoxygenase; HETE, hydroxy eicosatetraenoic acid; HPETE, hydroperoxyl eicosatetraenoic acid; HDHA, hydroxy docosahexaenoic acid; HpDHA, hydroperoxy docosahexaenoic acid; PG, prostaglandin; TX, thromboxane; LT, leukotriene; LX, lipoxin; Rv, resolvin.

[11–13].

The omega-6 PUFA arachidonic acid (AA, C20:4, n − 6) is subjected to enzymatic conversion to prostaglandins, leukotrienes, or lipoxins [14,15]. Omega-3 PUFAs eicosapentaenoic acid (EPA, 20:5n − 3) and docosahexaenoic acid (DHA, 22:6n − 3) are enzymatically converted into specific bioactive compounds that display protective anti-inflammatory and proresolving activities. PUFA enzymatic reactions are mediated by cyclooxygenases (COX) and lipoxygenases (LO), as depicted in Fig. 1 [16–19]. Prostaglandins and leukotrienes amplify the production of proinflammatory cytokines, whereas specialized proresolving mediators, including lipoxins, resolvins, and protectins, modulate immune responses to control local inflammation.

Resolvins RvD1 and RvE1 display protective actions in mouse models of pneumonia and acute lung injury by reducing lung levels of proinflammatory cytokines such as IL-1β, IL-6 and TNF-α, enhancing bacterial clearance, decreasing lung neutrophil trafficking, and improving survival [20,21]. In addition to cytokines, prostaglandin E2 (PGE2) and leukotriene B4 (LTB4) regulation, resolvins RvD2 and RvE1 and aspirin-triggered lipoxin potentiate the activation of antimicrobial mechanisms, *i.e.*, phagocytosis, reactive oxygen species (ROS) production and the induction of bactericidal/permeability-increasing protein (BPI), to protect mucosae from Gram-negative infections [20,22–26]. The specialized proresolving mediators are also antagonists of proinflammatory mediators in chronic inflammatory processes [27].

The role that resolution of inflammation plays during infection with *M. tuberculosis* is unknown but may be relevant to the pathogenesis of the disease. Production of PGE2 and LTB4 has been reported to be detrimental in a murine model of progressive tuberculosis, and blockade of PGE2 synthesis correlates with infection control and inflammation resolution [28]. However, *in vitro* models indicate that the virulent strain *M. tuberculosis* H37Rv reduces PGE2 synthesis and induces the production of lipoxin A4 (LXA4) in infected macrophages to promote necrosis and ensure bacterial dissemination [29]. The role of other specialized proresolving mediators during *M. tuberculosis* infection remains to be elucidated.

Because the resolution of inflammation appears to be necessary in *M. tuberculosis* infection, it is likely that lipoxins, resolvins, and protectins may have a substantial role in the infection that has not been characterized. Hence, in this study, we evaluated the effects of the addition of resolvins (RvD1 and RvD2), protectins (PDX and Mar1), and lipoxin (LXA4) on the inflammatory microenvironment and the antimicrobial activity of human macrophages infected with *M. tuberculosis* H37Rv.

## 2. Methods

### 2.1. Reagents

Bioactive compounds, omega-3 and 6 PUFAs, 17(*S*)-resolvin D1 (RvD1), 7(*S*),16(*R*),17(*S*)-resolvin D2 (RvD2), 5(*S*),6(*R*)-lipoxin A4 (LXA4), 10(*S*),17(*S*)-diHDoHE (PDX), and 7(*R*)-maresin 1 (Mar1), were purchased from Cayman Chemical (Ann Arbor, MI). Reagents were dissolved in ethanol (J.T. Baker, Avantor, Roadnor, PA). The culture proportions of ethanol were lower than 0.1%. Lipopolysaccharide (LPS) and all other reagents were purchased from Sigma Aldrich (St. Louis, MO), unless otherwise stated.

### 2.2. Cells

Monocytes were isolated from peripheral blood mononuclear cells by positive selection using magnetic beads (Miltenyi Biotech, Auburn, CA) as previously reported [8]. Cell suspension purity and viability were assessed by flow cytometry, revealing that 97.38 ± 1.26% of cells were CD14+ and that the cell viability was 99.4 ± 0.2% (mean ± SE). The monocyte suspension was adjusted to $1 \times 10^6$ cells/mL in RPMI-1640 supplemented with 200 mM L-glutamine (Lonza, Walkersville, MD), 5 μg $L^{-1}$ gentamicin sulfate (Lonza) and 10% heat-inactivated human serum (Valley Biomedicals, Winchester, VA). We cultured human monocytes for 7 days under adherence conditions to generate monocyte-derived macrophages (MDMs), herein referred to as macrophages. These studies were approved by the Institutional Review Board at the Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas (number B1615).

### 2.3. Assessment of proresolution mediators' effect on reduction of TNF-α production

Macrophages ($5 \times 10^5$ cells/well) were stimulated with 200 ng/mL of LPS for 1 h. Furthermore, RvD1, RvD2, PDX, LXA4 and Mar1 were added at varying concentrations, and the cells were cultured for an additional 24 h. The supernatants were collected and cryopreserved for TNF-α measurement. Viability was assessed using a CellTiter cell

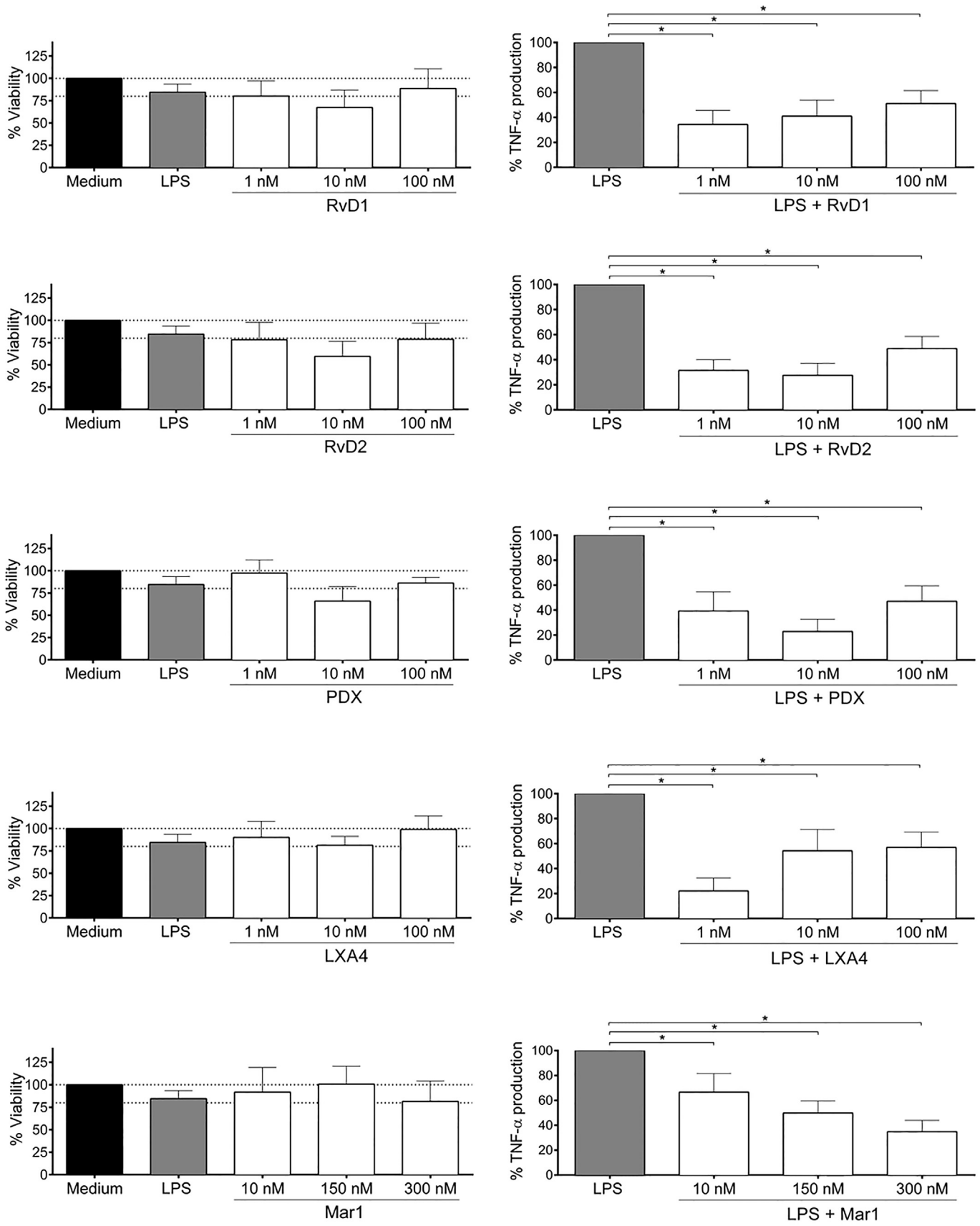


**Fig. 2.** Anti-inflammatory activity of the lipid resolvers of inflammation on macrophages stimulated with LPS. Cells were stimulated with 200 ng/mL LPS for 1 h, and then RvD1, RvD2, PDX, or LXA4 (at 1, 10 and 100 nM) or Mar1 (at 10, 150, and 300 nM) was added and incubated for another 24 h. The supernatants were collected, and TNF-α production was measured by ELISA (right). Cell viability was determined in the presence of the lipids alone (left). Depicted are the means ± standard errors, n = 7. $^{*}p < 0.05$.

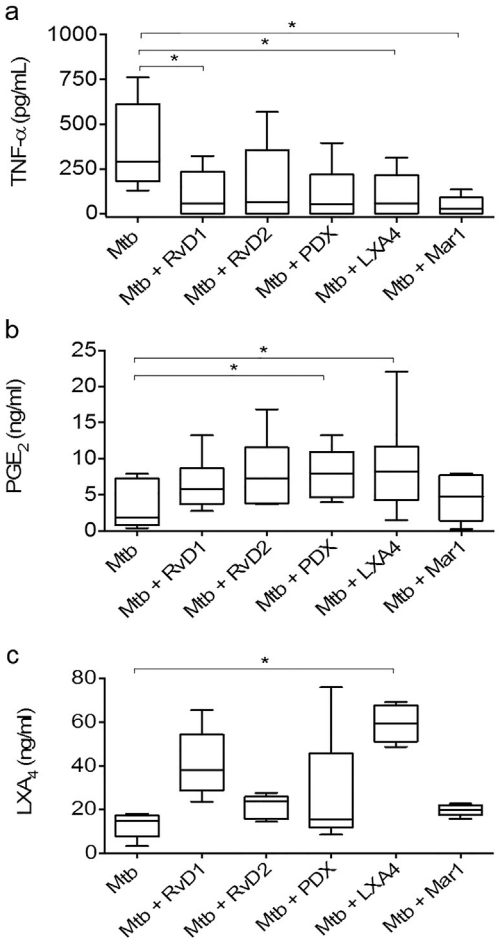


**Fig. 3.** Effects of the lipids under study on the production of TNF-α, PGE2 and LXA4. Macrophages were infected with *M. tuberculosis* H37Rv (Mtb) at an MOI of 5 for 1 h. Nonphagocytosed bacteria were removed, RvD1, RvD2, PDX, or LXA4 (100 nM) or Mar1 (150 nM) was added, and the cells were incubated. After 24 h, the supernatants were recovered for the quantification of a) TNF-α (n = 14) by ELISA and of b) PGE2 (n = 10) and c) LXA4 (n = 5) by EIA. Box plots indicate the medians and quartiles. *$p < 0.05$ *vs* Mtb alone.

viability assay (Promega, Madison, WI, USA) probing the lipids in the same concentrations but in the absence of LPS.

### 2.4. Mycobacteria preparation

*M. tuberculosis* H37Rv (ATCC, Manassas, VA) was cultured in Middlebrook 7H9 broth medium (Difco Laboratories, Detroit, MI). After 21 days of incubation of *M. tuberculosis* at 37 °C, the mycobacterial stock solution was harvested, aliquoted and stored at −80 °C until use. Colony-forming units (CFUs) were determined after disruption of mycobacterial clumps [30].

### 2.5. Infection with M. tuberculosis and treatment with the resolvins RvD1 and RvD2, the protectins PDX and Mar1 and the lipoxin LXA4

Macrophages ($2 \times 10^5$ per well) were infected with *M. tuberculosis* at an MOI of 5 in RPMI with 30% non-heat-inactivated, pooled human AB serum. 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 100 nM resolvin D1 (RvD1), resolvin D2 (RvD2), protectin PDX or lipoxin LXA4, or 150 nM maresin 1 (Mar1). The cells were cultured for 24 h before collection of the supernatants and cell lysates, which were stored at −20 °C until use. We included cells treated only with LPS (200 ng/mL) as a control.

### 2.6. Antimicrobial activity

To evaluate the effects of the specialized proresolving mediators on mycobacterial intracellular growth control, macrophages were infected at an MOI of 5 and treated with the same amounts of the specialized proresolving mediators described above after extensive washing to remove nonphagocytosed bacteria. Macrophages infected with *M. tuberculosis* were further incubated at 37 °C in 5% $CO_2$ for 1 h (day 0) and 72 h (day 3). After incubation, the supernatants were discarded, and the cells were lysed with 0.1% SDS for 10 min and then neutralized with 20% bovine serum albumin (BSA). The lysates were serially diluted and plated onto 7H10 (Difco Laboratories) agar plates in triplicate. The CFUs were quantified after 21 days.

### 2.7. Cytokine production

Supernatants from 24 h cultures were assayed for the release of IL-6, IL-8, IL-10 and GM-CSF using Bio-Plex human cytokine customized detection kits (Bio-Rad, Hercules, CA, US) following the manufacturer's protocol. In selected experiments, TNF-α and IL-10 levels were measured using an in-house enzyme-linked immunosorbent assay (ELISA) with specific pairs of antibodies, including anti-human TNF-α (Pharmingen, San Diego, CA) or anti-IL-10 (Probiotek, Monterrey, MX), as previously described [31]. 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.

### 2.8. Prostaglandin E2 and lipoxin A4 detection

Prostaglandin E2 and lipoxin A4 production in the culture supernatants was quantified by means of an enzymatic immunoassay (EIA). Each sample was assayed in duplicate using prostaglandin E2 and lipoxin A4 EIA kits (Oxford Biomedical Research, Oxford, MI) according to the manufacturer's instructions.

### 2.9. Reverse transcription and real-time PCR for gene expression

Total RNA was extracted and reverse transcribed; the cDNA was subjected to quantitative real-time PCR (qRT-PCR, TaqMan) to determine the mRNA expression levels using the comparative threshold cycle (ΔΔCt), as previously described [6]. Real-time PCRs were performed in duplicate wells according to the manufacturer's protocol for TaqMan predesigned gene assays. The LL37 (Hs00189038_m1) and BPI (Hs01552756_m1) genes were evaluated, and gene assays were purchased from Applied Biosystems (Carlsbad, CA). The Ct values for each gene were normalized to the endogenous control 18S rRNA gene (4319413E).

### 2.10. Extraction of nuclear proteins

The macrophages were cultured, $5 \times 10^6$ cells per experimental condition, in ultralow attachment plates (Corning Incorporated, Corning, NY). The cells were stimulated with LPS (500 ng/mL) or with 100 nM RvD1 or 150 nM Mar1 for 60 min. The cells were detached from the plates with 1 mM EDTA, and the viability was checked by trypan blue exclusion. The cells were transferred to 14 mL polypropylene tubes and centrifuged for 5 min at 1500 rpm at 4 °C, and the nuclear proteins were extracted. All procedures were performed in cold conditions. The supernatant was discarded, the cell pellet was transferred in 1 mL of phosphate-buffered saline (PBS) to a 1.5 mL tube and washed three times with cold PBS (5 min at 3500 rpm at 4 °C), and the supernatant was completely removed. The cell pellet was resuspended in a hypotonic buffer to obtain the cytoplasmic extract (20 mM HEPES [4-(2-

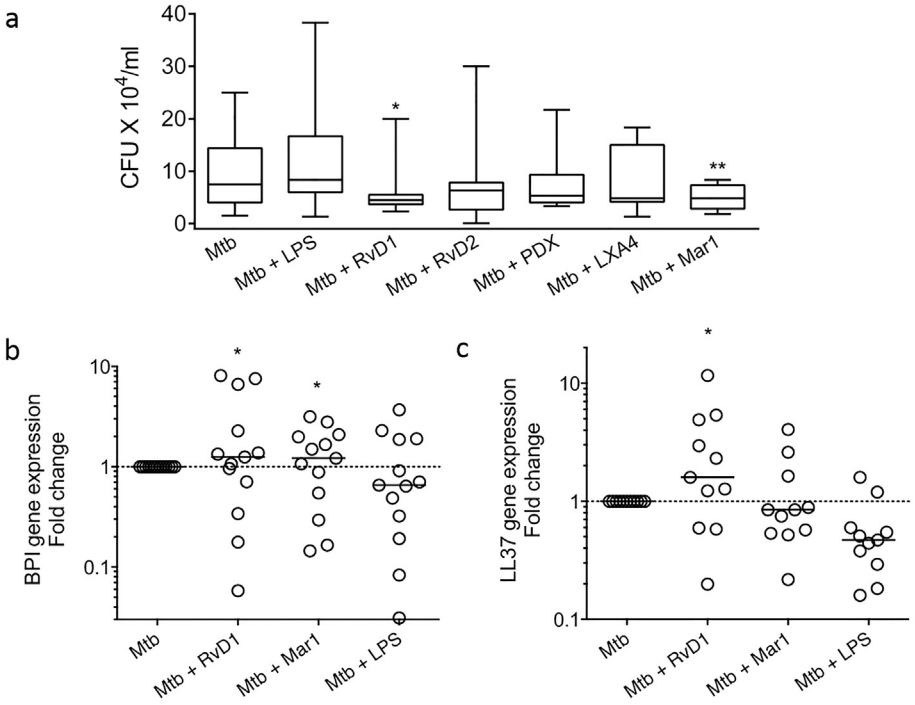


**Fig. 4.** Effects of lipids on bactericidal activity and BPI and LL37 expression by macrophages infected with *M. tuberculosis*. MDMs were infected with *M. tuberculosis* H37Rv (Mtb) at an MOI of 5 for 1 h. Nonphagocytosed bacteria were removed, and 200 ng/mL LPS, 100 nM RvD1, RvD2, PDX, or LXA4 or 150 nM Mar1 was added to the culture. a) The intracellular bacterial load was determined by the colony-forming unit (CFU) method after 3 days of culture. Box plots indicate the medians and quartiles, n = 11. $^*p < 0.05$. Cell lysates were used for the quantification of gene expression of b) BPI (n = 13) and c) LL37 (n = 11) by real-time PCR (qPCR). Gene expression was normalized to the relative abundance of 18S RNA. Depicted are individual results of the fold change relative to infection alone, using the method of comparative Ct. Lines indicate the medians. $^*p < 0.05$.

hydroxyethyl)-1-piperazineethanesulfonic acid] pH 7.9, 400 mM NaCl [sodium chloride], 1.5 mM $MgCl_2$ [magnesium chloride], 25% glycerol, 0.2 mM EDTA, 1 mM fresh DTT [dithiothreitol] and 0.5 mM fresh PMSF [phenylmethylsulfonyl fluoride]) and left for 1 min on ice before centrifuging at 3500 rpm for 10 min at 4 °C. The cytoplasmic fraction (supernatant) was cryopreserved. The pellet was resuspended in 20 μL of hypertonic buffer (10 mM HEPES pH 7.0, 10 mM KCl [potassium chloride], 1.5 mM $MgCl_2$, and 1 mM fresh DTT) and incubated for 30 min with gentle agitation at 4 °C. The pellet was then centrifuged at 14,000 rpm for 20 min at 4 °C, and the supernatant containing the nuclear protein was recovered and stored at −20 °C until use. The samples were validated by western blotting *via* the expression of proliferating cell nuclear antigen (PCNA, Supplementary Fig. 1).

### 2.11. Quantification of Nrf2 in nuclear extracts

To assess the nuclear translocation of Nrf2, the nuclear and cytosolic extracts were assayed using a TransAM Nrf2 kit (Active Motif, Carlsbad, CA) following the manufacturer's instructions.

### 2.12. NFκB translocation

The nuclear translocation of NFκB p65 was determined by imaging cytometry of intact cells. Macrophages were stimulated with LPS (500 ng/mL), 100 nM RvD1 or 150 nM Mar1 for 60 min. The cells were immediately washed with ice-cold PBS (Lonza) and stained using an Amnis NFκB Translocation kit (Millipore, Burlington, MA) following the manufacturer's instructions. The cells were immediately analyzed using Image Stream MKII equipment (Amnis Corporation, Seattle, WA). At least 1000 cells were photographed, and nuclear translocation was measured using the Nuclear Localization Wizard within the Amnis Ideas software (Amnis Corporation). A similarity index above 1.2 determined the nuclear localization of NFκB. The similarity index is the log-transformed Pearson's correlation coefficient and is a measure of the degree to which two images are linearly correlated within a masked region. The analysis strategy is depicted in Supplementary Fig. 2. The results are expressed as the percentage of cells with nuclear NFκB translocation.

### 2.13. BPI detection by fluorescence microscopy

For fluorescence microscopy experiments, $2 \times 10^5$ cells/well were cultured in 8-well Lab-Tek II chambers. Macrophages were infected with *M. tuberculosis* at an MOI of 5. After discarding nonphagocytosed bacteria, the cells were incubated with the high-affinity D1-like antagonist hydrobromide (5 μM) (SCH, TOCRIS Bioscience, Bristol, UK) for 30 min prior to stimulation with RvD1 or Mar1 and were then incubated for an additional 24 h. The cells were fixed with 4% paraformaldehyde and stained with purified rabbit anti-hBPI (R&D systems, Minneapolis, MN) and rabbit anti-*M. tuberculosis* antibodies and appropriate secondary antibodies coupled with Alexa Fluor 488 and Alexa Fluor 594 (Life Technologies). Hoechst (Enzo Life Sciences, Plymouth Meeting, PA) was used to detect nuclei. The cells were visualized with a fluorescence Axio Scope A1 microscope (Carl Zeiss, Oberkochen, DE), and the images were acquired and analyzed with ZEN Pro software (Carl Zeiss). At least 50 cells were imaged for each condition. The cells were contoured with the Spline tool using the phase contrast image as the source. The selected region of interest was pasted onto the fluorescence image, and the mean fluorescence intensity of BPI per cell was calculated. The analysis strategy is depicted in Supplementary Fig. 3.

### 2.14. 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 statistically significant.

## 3. Results

### 3.1. Exogenous RvD1, RvD2, PDX, LXA4 and Mar1 modulate LPS-dependent TNF-α production

Specialized proresolving mediators are produced by macrophages and exert their biological function mainly on neutrophils. The effects of RvD1 and Mar1 on macrophages are poorly characterized. We first confirmed that the lipids in this study exerted modulatory activities on macrophages by measuring their effect on TNF-α production after LPS

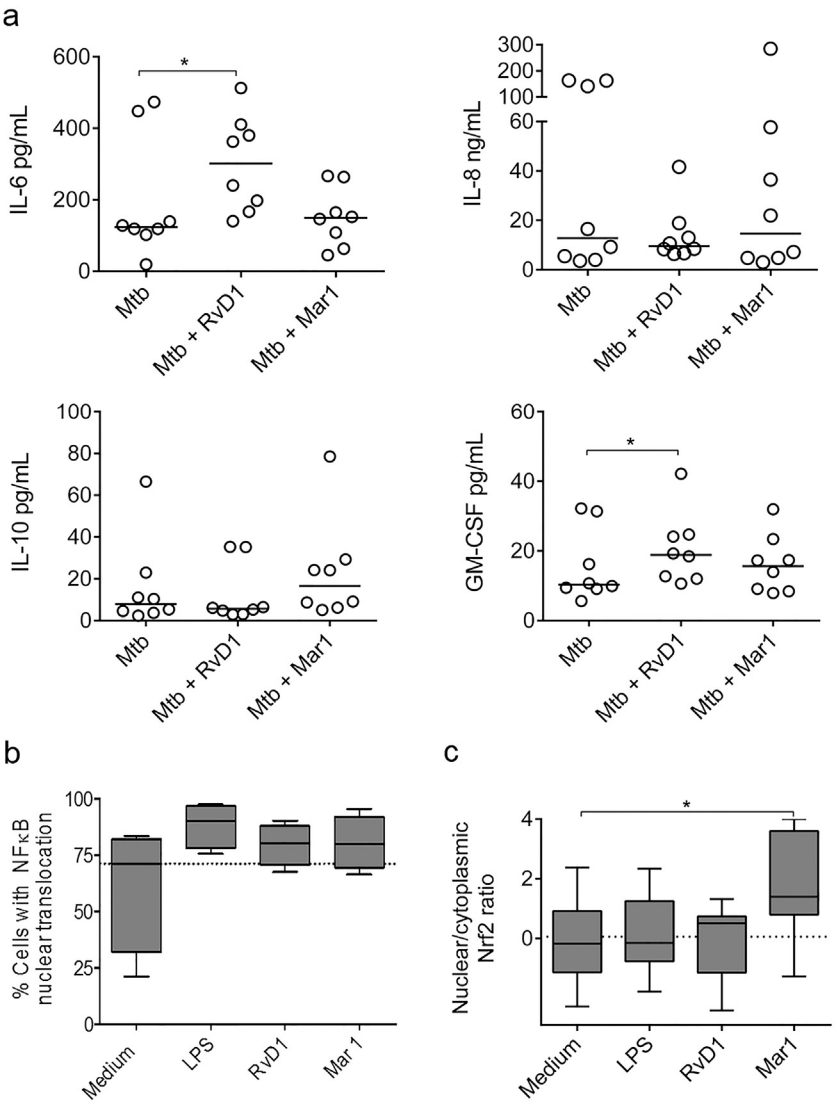


**Fig. 5.** Effects of RvD1 and Mar1 on the production of cytokines by macrophages infected with *M. tuberculosis*. MDMs were infected with *M. tuberculosis* H37Rv (Mtb) at an MOI of 5 for 1 h. Nonphagocytosed bacteria were removed, and 100 nM RvD1, 150 nM Mar1 or 200 ng/mL LPS was added. The cells remained in culture for 24 h, and a) the production of IL6, IL8, IL10 and GM-CSF was determined by Luminex. Depicted are individual results. Lines indicate the medians, n = 8. $^*p < 0.05$ between the indicated pairs of samples. Nuclear translocation of transcription factors was determined in uninfected cells stimulated with LPS, RvD1 or Mar1 after 2 h of culture. b) Nuclear translocation of NFκB was determined by imaging cytometry of intact cells. Depicted are the percentages of cells with translocation, n = 5. c) Nuclear translocation of Nrf2 was measured by ELISA in nuclear and cytoplasmic extracts of the cells. Depicted is the nuclear/cytoplasmic ratio. Box plots indicate the medians and quartiles, n = 8. $^*p < 0.05$ between the indicated pairs of samples.

stimulation. Based on the concentrations reported in the literature, we used 1, 10 and 100 nM for RvD1, RvD2, PDX, and LXA4 and 10, 150, and 300 nM for Mar1 to assess their ability to reduce the production of TNF-α after LPS stimulation in macrophages, and no significant reduction in cell viability was observed at any of the evaluated concentrations of each lipid (Fig. 2, left). We observed a non-dose-response significant reduction of TNF-α production by every specialized proresolving mediator (Fig. 2, right). Since there was no significant effect of the lipid concentration used, all subsequent experiments were performed using the highest concentration that did not affect the viability of the macrophages.

### 3.2. RvD1, RvD2, PDX, LXA4 and Mar1 modulate TNF-α production by macrophages infected with M. tuberculosis

We further evaluated the effect of specialized proresolving mediators on macrophage resolution during *M. tuberculosis* infection by measuring TNF-α, PGE2 and LXA4 production. We observed that RvD1, LXA4 and Mar1 were able to significantly reduce TNF-α production after *M. tuberculosis* infection (Fig. 3a). In addition, PDX and LXA4 induced a significant increase in PGE2 production in macrophages infected with *M. tuberculosis* (Fig. 3b) and LXA4 induced more LXA4 production (Fig. 3c), but we could not rule out the possibility that the amounts of LXA4 in LXA4-stimulated cells were remnants of the exogenously applied lipoxin instead of autoinduced production.

### 3.3. RvD1 and Mar1 enhance the antimicrobial activity of macrophages infected with M. tuberculosis H37Rv

Because resolution of inflammation may have a role in antimicrobial responses, we investigated the role that inflammation resolution plays in the control of *M. tuberculosis* infection. We observed a significant reduction in the intracellular bacterial burden in macrophages stimulated with RvD1 (median 4.5 [range 2.3–20] $\times 10^4$ CFU/mL) and Mar1 (median 4.8 [range 1.8–8.3] $\times 10^4$ CFU/mL) compared to that in untreated macrophages (median 7.5 [range 1.5–25] $\times 10^4$ CFU/mL) (Fig. 4a). The other lipids exhibited no effect on bacterial burden control.

Because RvD1 and Mar1 activated the macrophage antimicrobial response, we evaluated whether this effect was associated with the induction of antimicrobial peptides. LPS was included because it induces inflammation but dampens antimicrobial responses. We observed that both RvD1 and Mar1 induced the overexpression of BPI in infected macrophages, with a median fold change of 2.276 [range 0.05–8.1] and a median fold change of 1.665 [range 0.14–3.157], respectively, relative to the expression of BPI in untreated cells (Fig. 4b). LPS did not induce BPI upregulation. Overexpression of LL37 was observed after RvD1 stimulation, with a median fold change of 1.59 [range

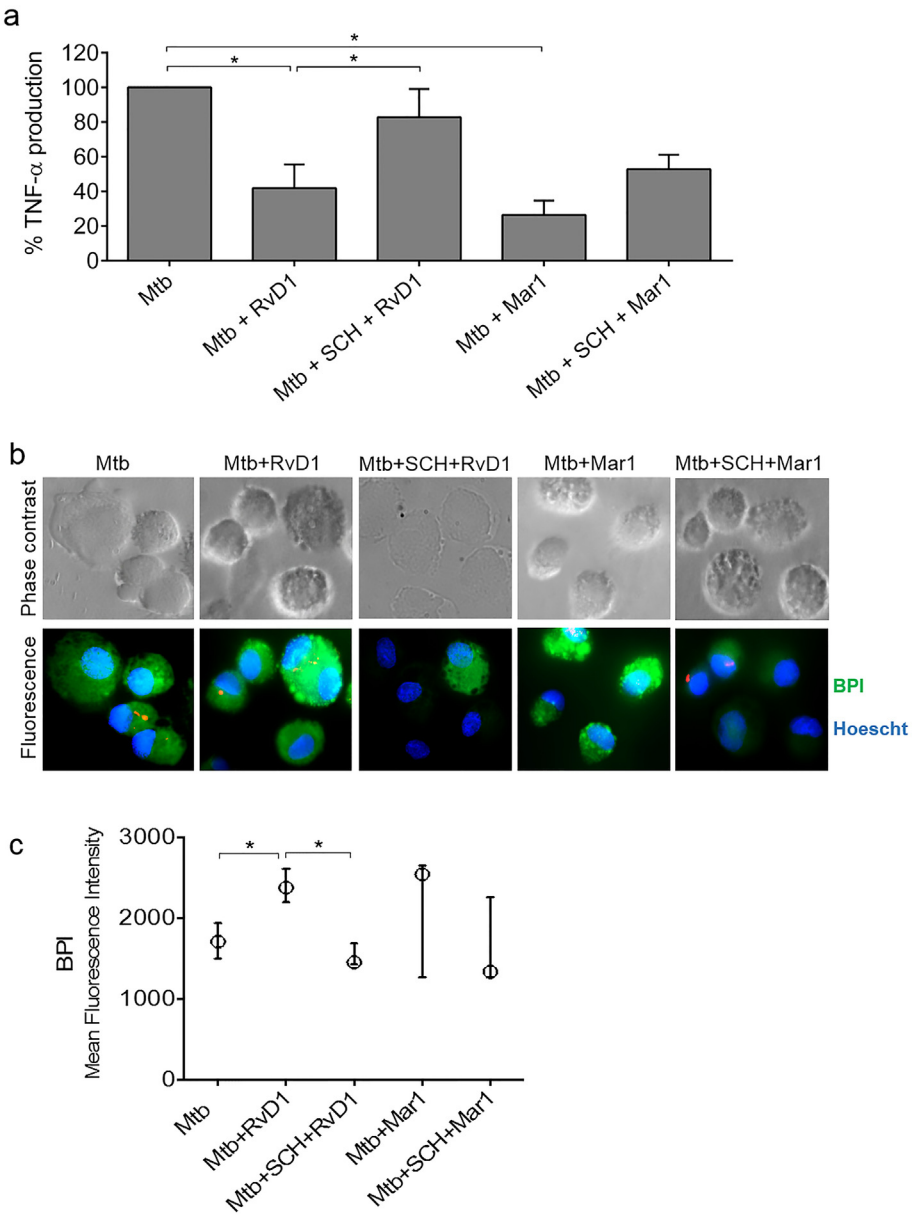


**Fig. 6.** A high-affinity D1-like antagonist inhibited the effects of RvD1 and Mar1 on the modulation of TNF-α production and decreased the BPI production induced by RvD1 in macrophages infected with *M. tuberculosis*.
MDMs were infected with *M. tuberculosis* H37Rv (Mtb) at an MOI of 5 for 1 h. Nonphagocytosed bacteria were removed. The high-affinity D1-like antagonist hydrobromide (SCH, 5 μM) was added 30 min before the addition of RvD1 (100 nM) or Mar1 (150 nM). The cells were incubated for another 24 h. a) The supernatants were collected, and TNF-α production was measured by ELISA. Depicted are the means ± standard errors, n = 6. $^*p < 0.05$ between the indicated pairs of samples. The cells were fixed, and BPI protein was detected by fluorescence microscopy. b) Representative images of phase contrast and fluorescence staining for the experimental conditions. Purified rabbit anti-hBPI (green) and rabbit anti-*M. tuberculosis* (red) antibodies were used and counterstained with appropriate Alexa Fluor-conjugated secondary antibodies. Hoechst (blue) was used to stain nuclei. c) At least 50 cells were imaged for each condition, and the mean fluorescence intensity of BPI per cell was calculated. The medians with interquartile ranges are depicted, n = 6. $^*p < 0.05$ between the indicated pairs of samples. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

0.19–11.64] (Fig. 4c). Apparently, the addition of LPS to infected macrophages downregulated BPI and LL37 expression.

### 3.4. Differential cytokine responses elicited by RvD1 and Mar1 in infected macrophages

The distinct antimicrobial responses induced by RvD1 and Mar1 prompted us to determine whether they induce differential cytokine responses. We found that RvD1 induced the production of significant amounts of IL-6 and GM-CSF, but not of IL-10 or IL-8, in macrophages infected with *M. tuberculosis*, whereas Mar1 did not induce a significant increase in the production of any of the cytokines (Fig. 5a). Neither the cytokine production nor the previous effects observed for RvD1 and Mar1 were due to the vehicle (Supplementary Fig. 4).

Because proinflammatory cytokine and antimicrobial peptide induction requires transcription signals to translocate to the nucleus, we determined the translocation of the nuclear factors NFκB p65 (inducer of the transcription of proinflammatory cytokines and antimicrobial peptides) and Nrf2 (nuclear factor that activates the transcription of anti-inflammatory molecules). For these experiments, LPS, RvD1 and Mar1 were used as single stimulants to prevent the effect of infection. We observed that both RvD1 and Mar1 induced the translocation of NFκB p65 to the nucleus in a smaller proportion than LPS (Fig. 5b), but only Mar1 induced a significant translocation of Nrf2 in comparison to macrophages without stimulation (Fig. 5c).

### 3.5. A high-affinity D1-like antagonist inhibits the effects of RvD1 and Mar1 on TNF-α and BPI production in macrophages infected with M. tuberculosis

We explored the role of G protein-coupled receptor (GPCR) signaling in RvD1 and Mar1 modulation of macrophage responses during *M. tuberculosis* infection. We used the D1-like antagonist SCH prior to RvD1 and Mar1 stimulation. We found that the addition of SCH to infected macrophages abrogated the regulatory effect of RvD1 on TNF-α production (Fig. 6a). Moreover, the addition of SCH inhibited the overexpression of BPI induced by RvD1 (Fig. 6b and Fig. 6c). Conversely, inhibition of GPCR signaling only partially reversed Mar1 effects.

## 4. Discussion

The immune response to *M. tuberculosis* induces a bactericidal and proinflammatory response that is important to control the infection, but at the same time, the induced inflammatory response is responsible for tissue injury during the tuberculosis disease process. Therefore, treatment of tuberculosis should include the addition of anti-inflammatory therapies to prevent tissue damage associated with necrosis during respiratory tract infections.

In this study, we evaluated the ability of proresolving mediators to mediate the regulation of TNF-α production and bactericidal responses in *M. tuberculosis*-infected macrophages. Proresolving mediators are typically implicated in the resolution of acute inflammation. They are released early to the extracellular space and are relevant for infections with gram-negative pathogens and influenza virus. The implication of these mediators in chronic infections, however, is unknown.

First, we demonstrated that RvD1, RvD2, PDX, LXA4 and Mar1 reduced TNF-α production after LPS stimulation in human macrophages. These results are in accordance with previous reports describing the protective effect of RvD1 on LPS-induced TNF-α production in mice [21]. However, the modulation of TNF-α production by RvD2, PDX, LXA4 and Mar1 in human macrophages had not been described before.

We also observed that RvD1, LXA4 and Mar1 significantly reduced TNF-α production in *M. tuberculosis*-infected macrophages and exhibited a modulatory effect similar to that observed with LPS stimulation. In addition, PDX and LXA4 induced a significant increase in PGE2 production in *M. tuberculosis*-infected macrophages; PGE2 is important in inflammation resolution [32] and plays a role in controlling necrosis during mycobacterial infection [29]. Therefore, the addition of PDX or LXA4 during mycobacterial infection could decrease necrosis through the induction of PGE2. However, it has been reported that in macrophages infected with virulent *M. tuberculosis*, LXA4 acts by downregulating PGE2 synthesis, which is in contrast with our observation [29]. These inconsistent results could be associated with the fact that PGE2 exhibits both anti- and proinflammatory effects depending on the concentration and timing of culture [33,34], and PGE2 induction during *M. tuberculosis* infection not only could be beneficial for necrosis control but may also induce a proinflammatory response.

In addition, the lack of regulation by RvD1, RvD2 and Mar1 exerted on PGE2 and LXA4 production suggests differential mechanisms of inflammation regulation by the proresolving mediators in macrophages. Except for LXA4, no role for the other proresolving mediators has been identified in the *M. tuberculosis* infection.

Interestingly, the addition of RvD1 and Mar1 also induced a significant reduction in *M. tuberculosis* intracellular growth that was accompanied by a significant overexpression of LL37 and BPI in RvD1-treated macrophages and only BPI in Mar1-treated macrophages. Antimicrobial responses have been reported to be induced by proresolving mediators in animal models. For instance, it has been described in animal models of pneumonia that treatment with the E-series resolvin RvE1 or aspirin-triggered RvD1 enhances bacterial clearance while decreasing lung levels of proinflammatory cytokines [35,36]. However, the antimicrobial mechanisms induced by these lipids are poorly characterized, and the induction of LL-37 and BPI by RvD1 and Mar1 in human macrophages has not been previously reported. Thus far, the induced-antimicrobial mechanism described for resolvins is the production of ROS [26]; meanwhile, it has been reported that aspirin-activated lipoxin (ATL) induces the expression of BPI in epithelial cells [37].

This study is the first report in which BPI and LL-37 are associated with the induction of antimicrobial responses by resolvins and maresins. It has been described that BPI plays a role in modulating TNF-α responses [38], and our results imply that the induction of BPI by RvD1 and Mar1 may exert dual effects (antibacterial and anti-inflammatory) during *M. tuberculosis* infections.

We wondered if the differential response that we observed for RvD1 and Mar1 in antimicrobial effector induction extended to the production of other cytokines. We observed that the addition of RvD1 and Mar1 to infected macrophages induced a differential profile of cytokine production. While RvD1 induced a significant increase in the amounts of IL-6 and GM-CSF, Mar1 did not induce an increased production of any of the cytokines. Therefore, we hypothesized that the differential cytokine production and the differential activation of bactericidal mechanisms induced by RvD1 and Mar1 can be explained by their differential ability to induce nuclear translocation of both proinflammatory (NFκB p65) and anti-inflammatory (Nrf2) transcription factors. Although both RvD1 and Mar1 induced a similar nuclear translocation of NFκB p65, only Mar1 mobilized a significant proportion of Nrf2 to the nucleus. Since activators of Nrf2 reportedly reduce Il-6 and IL-1 transcript accumulation in both murine bone marrow macrophages and a human macrophage cell line (THP-1) [39], these results suggest that Mar1 activates the Nrf2 pathway to reduce the production of anti-inflammatory cytokines, such as IL-6 and TNF-α. In addition, it has been described that RvD1 inhibits the inflammatory response through different mechanisms, such as induction of the nuclear translocation of NFκB p50/p50, which deactivates proinflammatory responses dependent on COX-2 [40]; reduction in the ratio of p-IκB/IkB, which reveals that RvD1 could inhibit the activation of NFκB [41]; and induction of IL-10 secretion, inactivation of GSK3, and activation of CREB to regulate inflammation in human monocytes stimulated with LPS [42]. These mechanisms have also been reported for RvD2 and Mar1. PGE2 also participates in the regulation of TNF-α production [43], but we did not observe induction of PGE2 by RvD1 or Mar1.

Finally, we investigated whether the RvD1- and Mar1-dependent responses were associated with GPCR signaling. We found that preincubation of infected macrophages with a D1-like antagonist (SCH) abrogated the regulatory effect of RvD1 on TNF-α production and the induction of BPI overexpression. However, SCH only partially altered the Mar1-dependent responses, suggesting a differential involvement of regulatory pathways that were beyond the reach of this study.

Overall, treatment with RvD1 and Mar1 induced an immunomodulation of *M. tuberculosis*-infected macrophages. Importantly, these proresolving mediators lack the immunosuppressive properties of classical anti-inflammatory therapies. Both proresolving mediators are metabolized from DHA. Essential DHA and EPA and their derivatives have been reported to be significantly reduced in the sera of tuberculosis patients [44], suggesting that patients with active tuberculosis may have low levels of resolvins. In addition, aspirin induces the production of RvD1 and Mar1 [35,45], which makes it possible to propose their pharmacological induction.

## 5. Conclusion

RvD1 and Mar1 induce anti-inflammatory mechanisms (regulation of TNF-α, PGE2 and other cytokines), differential production of antimicrobial effectors (BPI and LL37) and intracellular growth control of *M. tuberculosis* by human macrophages. These findings have clinical implications that need to be addressed in the future. In particular, the dietary origin of these lipids and their induction by nonsteroidal anti-inflammatory drugs such as aspirin and celecoxib make them potential targets of new therapeutic schemes in patients with pulmonary tuberculosis.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.105694.

## Acknowledgments

A. R. would like to thank the “Programa de Doctorado en Ciencias Biológicas,” Universidad Nacional Autónoma de México, and appreciates the scholarship received from “Consejo Nacional de Ciencia y Tecnología Mexico (CONACyT-Mexico)”.

## Declaration of Competing Interests

None declared.

## References


[1] World Health Organization, Global Tuberculosis Report 2018, (2018).
[2] C.R. Scharn, A.C. Collins, et al., Heme oxygenase-1 regulates inflammation and mycobacterial survival in human macrophages during Mycobacterium tuberculosis infection, J. Immunol. 196 (11) (2016) 4641–4649.
[3] A.M. Cooper, K.D. Mayer-Barber, et al., Role of innate cytokines in mycobacterial infection, Mucosal Immunol. 4 (3) (2011) 252–260.
[4] G.A. Rook, R. al Attiyah, et al., New insights into the immunopathology of tuberculosis, Pathobiology 59 (3) (1991) 148–152.
[5] S. Sharma, M. Bose, Role of cytokines in immune response to pulmonary tuberculosis, Asian Pac. J. Allergy Immunol. 19 (3) (2001) 213–219.
[6] E. Juarez, C. Carranza, et al., NOD2 enhances the innate response of alveolar macrophages to Mycobacterium tuberculosis in humans, Eur. J. Immunol. 42 (4) (2012) 880–889.
[7] E. Juarez, C. Carranza, et al., Nucleotide-oligomerizing domain-1 (NOD1) receptor activation induces pro-inflammatory responses and autophagy in human alveolar macrophages, BMC Pulm. Med. 14 (2014) 152.
[8] E. Juarez, C. Carranza, et al., Loperamide restricts intracellular growth of mycobacterium tuberculosis in lung macrophages, Am. J. Respir. Cell Mol. Biol. 55 (6) (2016) 837–847.
[9] C.N. Serhan, K. Gotlinger, et al., Resolvins, docosatrienes, and neuroprotectins, novel omega-3-derived mediators, and their aspirin-triggered endogenous epimers: an overview of their protective roles in catabasis, Prostaglandins Other Lipid Mediat. 73 (3–4) (2004) 155–172.
[10] J.M. Schwab, N. Chiang, et al., Resolvin E1 and protectin D1 activate inflammation-resolution programmes, Nature 447 (7146) (2007) 869–874.
[11] J.N. Fullerton, A.J. O'Brien, et al., Pathways mediating resolution of inflammation: when enough is too much, J. Pathol. 231 (1) (2013) 8–20.
[12] A. Ortega-Gomez, M. Perretti, et al., Resolution of inflammation: an integrated view, EMBO Mol. Med. 5 (5) (2013) 661–674.
[13] H. Andersson, B. Andersson, et al., Apoptotic neutrophils augment the inflammatory response to Mycobacterium tuberculosis infection in human macrophages, PLoS One 9 (7) (2014) e101514.
[14] B. Samuelsson, S.E. Dahlen, et al., Leukotrienes and lipoxins: structures, biosynthesis, and biological effects, Science 237 (4819) (1987) 1171–1176.
[15] C.N. Serhan, Resolution phase of inflammation: novel endogenous anti-inflammatory and proresolving lipid mediators and pathways, Annu. Rev. Immunol. 25 (2007) 101–137.
[16] P.C. Calder, The role of marine omega-3 (n − 3) fatty acids in inflammatory processes, atherosclerosis and plaque stability, Mol. Nutr. Food Res. 56 (7) (2012) 1073–1080.
[17] D.W. Gilroy, T. Lawrence, et al., Inflammatory resolution: new opportunities for drug discovery, Nat. Rev. Drug Discov. 3 (5) (2004) 401–416.
[18] C.N. Serhan, N. Chiang, et al., Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators, Nat. Rev. Immunol. 8 (5) (2008) 349–361.
[19] M. Spite, C.N. Serhan, Novel lipid mediators promote resolution of acute inflammation: impact of aspirin and statins, Circ. Res. 107 (10) (2010) 1170–1184.
[20] H. Seki, K. Fukunaga, et al., The anti-inflammatory and proresolving mediator resolvin E1 protects mice from bacterial pneumonia and acute lung injury, J. Immunol. 184 (2) (2010) 836–843.
[21] B. Wang, X. Gong, et al., Resolvin D1 protects mice from LPS-induced acute lung injury, Pulm. Pharmacol. Ther. 24 (4) (2011) 434–441.
[22] D. El Kebir, P. Gjorstrup, et al., Resolvin E1 promotes phagocytosis-induced neutrophil apoptosis and accelerates resolution of pulmonary inflammation, Proc. Natl. Acad. Sci. U. S. A. 109 (37) (2012) 14983–14988.
[23] D. El Kebir, L. Jozsef, et al., 15-Epi-lipoxin A4 inhibits myeloperoxidase signaling and enhances resolution of acute lung injury, Am. J. Respir. Crit. Care Med. 180 (4) (2009) 311–319.
[24] M. Morita, K. Kuba, et al., The lipid mediator protectin D1 inhibits influenza virus replication and improves severe influenza, Cell 153 (1) (2013) 112–125.
[25] C.D. Palmer, C.J. Mancuso, et al., 17(R)-Resolvin D1 differentially regulates TLR4-mediated responses of primary human macrophages to purified LPS and live E. coli, J. Leukoc. Biol. 90 (3) (2011) 459–470.
[26] M. Spite, L.V. Norling, et al., Resolvin D2 is a potent regulator of leukocytes and controls microbial sepsis, Nature 461 (7268) (2009) 1287–1291.
[27] H.M. Hsiao, T.H. Thatcher, et al., Resolvin D1 reduces emphysema and chronic inflammation, Am. J. Pathol. 185 (12) (2015) 3189–3201.
[28] J. Rangel Moreno, I. Estrada Garcia, et al., The role of prostaglandin E2 in the immunopathogenesis of experimental pulmonary tuberculosis, Immunology 106 (2) (2002) 257–266.
[29] M. Chen, M. Divangahi, et al., Lipid mediators in innate immunity against tuberculosis: opposing roles of PGE2 and LXA4 in the induction of macrophage death, J. Exp. Med. 205 (12) (2008) 2791–2801.
[30] C. Carranza, E. Juarez, et al., Mycobacterium tuberculosis growth control by lung macrophages and CD8 cells from patient contacts, Am. J. Respir. Crit. Care Med. 173 (2) (2006) 238–245.
[31] S.K. Schwander, M. Torres, et al., Enhanced responses to Mycobacterium tuberculosis antigens by human alveolar lymphocytes during active pulmonary tuberculosis, J. Infect. Dis. 178 (5) (1998) 1434–1445.
[32] C.A. Loynes, J.A. Lee, et al., PGE2 production at sites of tissue injury promotes an anti-inflammatory neutrophil phenotype and determines the outcome of inflammation resolution in vivo, Sci. Adv. 4 (9) (2018) eaar8320.
[33] I.C. Osma-Garcia, C. Punzon, et al., Dose-dependent effects of prostaglandin E2 in macrophage adhesion and migration, Eur. J. Immunol. 46 (3) (2016) 677–688.
[34] E.V. Tchetina, J.A. Di Battista, et al., Prostaglandin PGE2 at very low concentrations suppresses collagen cleavage in cultured human osteoarthritic articular cartilage: this involves a decrease in expression of proinflammatory genes, collagenases and COL10A1, a gene linked to chondrocyte hypertrophy, Arthritis Res. Ther. 9 (4) (2007) R75.
[35] A. Croasdell, S.H. Lacy, et al., Resolvin D1 dampens pulmonary inflammation and promotes clearance of nontypeable Haemophilus influenzae, J. Immunol. 196 (6) (2016) 2742–2752.
[36] O. Eickmeier, H. Seki, et al., Aspirin-triggered resolvin D1 reduces mucosal inflammation and promotes resolution in a murine model of acute lung injury, Mucosal Immunol. 6 (2) (2013) 256–266.
[37] G. Canny, O. Levy, et al., Lipid mediator-induced expression of bactericidal/permeability-increasing protein (BPI) in human mucosal epithelia, Proc. Natl. Acad. Sci. U. S. A. 99 (6) (2002) 3902–3907.
[38] T.G. Jahr, L. Ryan, et al., Induction of tumor necrosis factor production from monocytes stimulated with mannuronic acid polymers and involvement of lipopolysaccharide-binding protein, CD14, and bactericidal/permeability-increasing factor, Infect. Immun. 65 (1) (1997) 89–94.
[39] E.H. Kobayashi, et al., Nrf2 suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription, Nat. Commun. (7) (2016) 1–14.
[40] Y. Gao, H. Zhang, et al., Resolvin D1 improves the resolution of inflammation via activating NF-kappaB p50/p50-mediated cyclooxygenase-2 expression in acute respiratory distress syndrome, J. Immunol. 199 (6) (2017) 2043–2054.
[41] Y.L. Zhao, L. Zhang, et al., Resolvin D1 protects lipopolysaccharide-induced acute kidney injury by down-regulating nuclear factor-kappa B signal and inhibiting apoptosis, Chin. Med. J. (Engl.) 129 (9) (2016) 1100–1107.
[42] Z. Gu, G.J. Lamont, et al., Resolvin D1, resolvin D2 and maresin 1 activate the GSK3beta anti-inflammatory axis in TLR4-engaged human monocytes, Innate Immun. 22 (3) (2016) 186–195.
[43] V. Lehmmann, B. Benninghoff, et al., Tumor necrosis factor-induced activation of peritoneal macrophages is regulated by prostaglandin E2 and cAMP, J. Immunol. 141 (2) (1988) 587–591.
[44] J. Weiner III, S.K. Parida, et al., Biomarkers of inflammation, immunosuppression and stress with active disease are revealed by metabolomic profiling of tuberculosis patients, PLoS One 7 (7) (2012) e40221.
[45] C.N. Serhan, S. Hong, et al., Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals, J. Exp. Med. 196 (8) (2002) 1025–1037.