

Lipid mediators in innate immunity against tuberculosis: opposing roles of PGE₂ and LXA₄ in the induction of macrophage death

Minjian Chen,¹ Maziar Divangahi,¹ Huixian Gan,¹ Daniel S.J. Shin,¹ Song Hong,² David M. Lee,¹ Charles N. Serhan,² Samuel M. Behar,¹ and Heinz G. Remold¹

¹Department of Medicine and Division of Rheumatology, Immunology, and Allergy and ²Center for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesiology, Perioperative, and Pain Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115

Virulent *Mycobacterium tuberculosis* (*Mtb*) induces a maladaptive cytolytic death modality, necrosis, which is advantageous for the pathogen. We report that necrosis of macrophages infected with the virulent *Mtb* strains H37Rv and Erdmann depends on predominant LXA₄ production that is part of the antiinflammatory and inflammation-resolving action induced by *Mtb*. Infection of macrophages with the avirulent H37Ra triggers production of high levels of the prostanoid PGE₂, which promotes protection against mitochondrial inner membrane perturbation and necrosis. In contrast to H37Ra infection, PGE₂ production is significantly reduced in H37Rv-infected macrophages. PGE₂ acts by engaging the PGE₂ receptor EP2, which induces cyclic AMP production and protein kinase A activation. To verify a role for PGE₂ in control of bacterial growth, we show that infection of prostaglandin E synthase (PGES)^{-/-} macrophages in vitro with H37Rv resulted in significantly higher bacterial burden compared with wild-type macrophages. More importantly, PGES^{-/-} mice harbor significantly higher *Mtb* lung burden 5 wk after low-dose aerosol infection with virulent *Mtb*. These in vitro and in vivo data indicate that PGE₂ plays a critical role in inhibition of *Mtb* replication.

CORRESPONDENCE

Heinz G. Remold:
hremold@rics.bwh.harvard.edu

Abbreviations used: 5-LO, 5-lipoxygenase; AA, arachidonic acid; COX, cyclooxygenase; cPLA₂, cytosolic PLA₂; EP, E prostanoid; LC-MS-MS, liquid chromatography tandem mass spectrometry; MOI, multiplicity of infection; mPGES, microsomal prostaglandin E synthase; MPT, mitochondrial permeability transition; PI, propidium iodide; PI3K, phosphatidylinositol-3 kinase; PKA, protein kinase A; siRNA, small interfering RNA.

Tuberculosis is the predominant cause for mortality from chronic pulmonary bacterial infections worldwide causing nearly two million deaths yearly. Tuberculosis has become more serious as a consequence of the global AIDS epidemic and the emergence of multidrug-resistant *Mycobacterium tuberculosis* (*Mtb*), the causative agent of tuberculosis (1). Natural transmission of *Mtb* occurs predominantly via inhalation of aerosols containing small numbers of bacteria that are deposited in the distal airways of the lung (2). The pathogens are phagocytosed by pulmonary Mφ, which serve as a sanctuary for *Mtb* (3). Virulent *Mtb*, which

reside in Mφ, initially evade elimination by the immune system via preventing apoptosis and inhibiting maturation of the phagosome-lysosome organelle of the host Mφ (4, 5). The net effect is to diminish entry of bacterial proteins into the class II MHC antigen-processing pathway (4) and to create a protected intracellular milieu where bacilli remain metabolically active and replication competent (6). For Mφ infected with virulent H37Rv, necrosis characterized by cytolysis is the dominant form of cell death, which affords a protective milieu for *Mtb* (7, 8). Thus, subversion of cell death toward necrosis is of considerable advantage for the pathogen.

M. Divangahi and H. Gan contributed equally to this study. M. Chen's present address is Clinical Research Center, The First Affiliated Hospital, Guangxi Medical University, Nanning, Guangxi 530021, People's Republic of China. S. Hong's present address is Louisiana State University Neuroscience Center, New Orleans, LA 70112.

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A common mechanism of necrosis is induction of mitochondrial permeability transition (MPT), which is manifested as accelerated cell death with plasma membrane disintegration. It is thought that a pore opens in the inner mitochondrial membrane allowing water and other molecules to pass through. Opening of this permeability transition pore can be triggered by multiple stimuli and leads to dissipation of the mitochondrial inner membrane potential ($\Delta\Psi_m$) (9). Irreversible induction of MPT leads to mitochondrial damage associated with mitochondrial swelling and subsequent necrosis of the cell. In vitro M ϕ infected with virulent H37Rv causes the catastrophic irreversible MPT that commits the M ϕ to necrosis (10).

The active nature of this necrosis induction by virulent *Mtb* is clearly revealed after infection of M ϕ with mutants of *Mtb*. Although host M ϕ support growth of virulent *Mtb* (3), innate immune mechanisms are activated that limit pathogen survival after infection with *Mtb* mutants having altered virulence. These responses include induction of apoptosis, a slow cell death modality which leaves the plasma membrane intact and is observed after infection of M ϕ with attenuated *Mtb* (11–13). This M ϕ death modality limits exploitation of the intracellular growth environment through direct microbicidal effects and by sequestering bacilli in apoptotic bodies. Indeed, apoptosis both enhances antigen presentation by DC (14) and facilitates efficient pathogen killing (15–18).

Two pathways are described that lead to this highly regulated form of cell death (19). First, the extrinsic apoptotic pathway implicates binding of the ligands TNF and FasL to their receptors that trigger apoptosis (20). Second, the intrinsic apoptotic pathway involves the mitochondria, which release cytochrome *c* and other factors from the mitochondrial intermembrane space that promote apoptosis (21, 22).

We demonstrated previously that mitochondria play an essential role to determine whether *Mtb*-infected M ϕ undergo cytolytic necrosis or apoptosis (8). Apoptosis induced by the mitochondrial pathway commences with Ca^{2+} release from the ER that leads to an increase of Ca^{2+} in the mitochondria as well as translocation of BAX into the mitochondria and BAK activation (23). Permeabilization of the mitochondrial outer membrane then proceeds via formation of a proteolipid pore and subsequent escape of proteins from the mitochondrial intermembrane space into the cytosol. More specifically, upon mitochondrial outer membrane permeabilization proapoptotic factors, including cytochrome *c*, are released from the mitochondrial intermembrane space into the cytosol. There, cytochrome *c* forms a complex with Apaf-1, leading to activation of caspase-9 that, in turn, activates executioner caspases such as caspase-3, -6, and -7, which are instrumental in the induction of apoptosis (19, 24).

Infection with the attenuated *Mtb* H37Ra, which has a mutation in PhoP which inhibits ESX-1 function (25), predominantly prevents necrosis and leads to sequestration and decimation of the intracellular bacteria (8, 26).

Mtb-induced apoptosis and antimycobacterial activity of human M ϕ requires the activity of cPLA₂- γ , a group IV cytosolic PLA₂ (cPLA₂) (27) which catalyzes the release of arachidonic

acid (AA) from the sn-2 position of membrane phospholipids (28). AA and its functionally diverse and biologically active eicosanoid products have been implicated in the regulation of programmed cell death in several cell types (29, 30). The lipoxins are AA metabolites generated by 5- and 15-lipoxygenases (5- and 15-LO) (31). Lipoxins modulate chemokine and cytokine expression, stimulate monocyte trafficking, and enhance M ϕ engulfment of apoptotic leucocytes (32). However the role of 5-LO products of AA such as lipoxins and leukotriene B₄, which amplifies PMN chemotaxis and production of granule products (33), has not been elucidated in modulating the death modality of M ϕ .

Prostanoids are lipid mediators generated from AA by the enzymatic action of the cyclooxygenases (COX) 1 and 2 to form the intermediate PGH₂, with subsequent metabolism to specific prostanoid species (e.g., PGD₂, PGE₂, PGF_{2 α} , PGI₂, and thromboxane) by prostaglandin synthases (34, 35). The functions of these prostanoids are defined by an array of specific receptors. In the case of PGE₂, differential expression of four distinct EP receptors defines the intracellular pathways that are activated by PGE₂ and results in specific functions that might either promote or inhibit inflammation. For example, in the lung, PGE₂ either promotes vasodilatation and increases vascular permeability or increases bronchodilatation (36). A recent study describes a noninflammatory function of PGE₂, up- or down-regulation of programmed cell death, which indicates that the mechanisms of PGE₂ activation are complex and context dependent (37).

Mice with a deleted 5-LO gene have increased IL-12, IFN- γ , and NO synthase 2 levels compared with WT mice after pulmonary *Mtb* infection. 5-LO^{-/-} mice also have significantly lower *Mtb* burdens and survive longer than WT animals after respiratory *Mtb* infection (38). Importantly, administration of a stable LXA₄ analogue was sufficient to reverse the increased resistance of 5-LO^{-/-} mice to *Mtb* infection. The finding that *Mtb* infection significantly increases AA production via activation of cPLA₂- γ (27) and that 5 LO^{-/-} mice are significantly more resistant to *Mtb* infections (38), raised the question of whether eicosanoid production is involved in macrophage necrosis and inhibition of apoptosis induced by virulent *Mtb*. We hypothesize that stimulation of lipoxin by virulent *Mtb* inhibits macrophage apoptosis, promotes necrosis, and represents a mechanism that allows *Mtb* to evade elimination by the innate immune system. We therefore investigated how lipoxins and prostanoids affect the outcome of *Mtb*-driven M ϕ death with respect to apoptosis and necrosis as a novel pathogenic mechanism that regulates the innate immune response in *Mtb* infection.

RESULTS

Virulent *Mtb* trigger production of LXA₄ in M ϕ

We demonstrated that inhibition of cPLA₂ abrogates apoptosis in *Mtb*-infected M ϕ , which could be restored by reconstitution of AA, the predominant product of cPLA₂ (27). These findings suggest that products synthesized from AA, namely the eicosanoids, regulate programmed M ϕ death induced

by *Mtb*. Two groups of eicosanoids are of special interest because of their opposing effects, the prostanoids that are derived by the COX pathway (34) and the lipoxins, a distinct class of LO-derived products which promote termination of inflammation (39).

The identification of PGE₂ and LXA₄ in infected Mφ supernatants was established from the diagnostic ions present in their liquid chromatography tandem mass spectrometry (LC-MS-MS) spectra (Fig. 1 A). We extended these studies by performing ELISA assays on H37Rv- and H37Ra-infected Mφ.

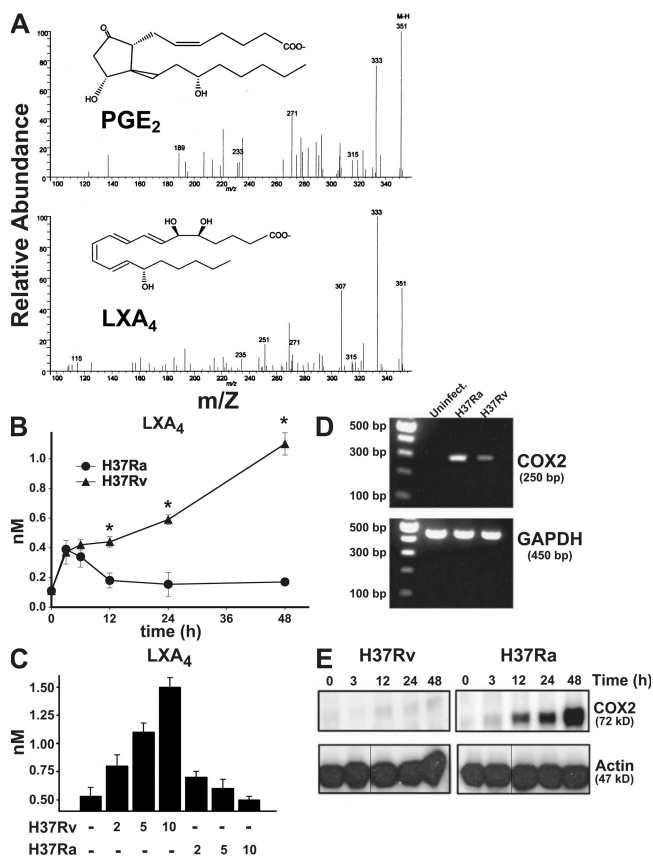


Figure 1. LXA₄ and COX2 production of human Mφ infected with H37Rv and H37Ra. (A) LC-MS-MS of endogenous PGE₂ (top) and LXA₄ (bottom) produced by H37Ra- (MOI 10) and H37Rv- (MOI 10) infected human Mφ, respectively, at 24 h. The spectrum is a representative LC-MS-MS ($n = 3$). The prominent ions and relative intensity matched with authentic PGE₂ and LXA₄ under these LC-MS-MS conditions. (B) LXA₄ production in human Mφ infected with H37Rv and H37Ra (MOI 10:1) at 0–48 h. Differences in LXA₄ concentrations in supernatants from H37Ra- and H37Rv-infected Mφ are statistically significant (*, $P < 0.002$; $n = 3$). (C) LXA₄ accumulation at 48 h in Mφ supernatants infected with H37Ra and H37Rv (MOI 2, 5, and 10:1). The differences in LXA₄ production induced by H37Ra and H37Rv are statistically significant at all MOIs ($P < 0.01$; $n = 3$). (D) COX2 mRNA accumulation in Mφ infected with H37Ra or H37Rv (MOI 10:1; uninfected, uninfected). (E) Production of COX2 protein in Mφ infected with H37Ra and H37Rv (MOI 10:1) at different time points. In all studies, n represents the number of independent experiments and the error bars represent SE. Black lines indicate that intervening lanes have been spliced out.

The production of LXA₄ was measured over time in supernatants from Mφ infected with virulent and avirulent *Mtb*. Virulent H37Rv induce significantly higher levels of LXA₄ production (1 nM; Fig. 1 B). Similar results are observed with another virulent strain (Erdmann strain [not depicted]). In contrast, when Mφ are infected with avirulent H37Ra, production of LXA₄ reaches ~0.4 nM at 4 and 6 h after infection but is then subsequently down-regulated to ~0.2 nM at 12 h. The amount of LXA₄ production induced by H37Rv correlates with the multiplicity of infection (MOI; Fig. 1 C). In contrast, the amount of LXA₄ production after H37Ra infection was not increased when the MOI was raised.

These findings raised the question of whether Mφ infected with virulent *Mtb* negatively regulate COX2 expression. Infection of Mφ with the virulent H37Rv induced little accumulation of COX2 mRNA and COX2 protein compared with infection with the mutant *Mtb* strain H37Ra, suggesting that H37Rv infection inhibits COX2 expression (Fig. 1, D and E). These findings suggest that infection with virulent *Mtb* induces the production of LXA₄ in sufficient quantities to inhibit COX2 production, which represents an important manifestation of bacterial virulence.

The virulent *Mtb* strain H37Rv inhibits prostanoid production by the host Mφ

COX2, the inducible PGH synthase, is required for prostanoid synthesis (40). To study whether inhibition of COX2 expression by H37Rv had any functional consequences, we measured prostanoid production by *Mtb*-infected Mφ. PGE₂ levels of <1 nM were detected in supernatants of H37Rv-infected Mφ during the first 48 h of infection. In contrast, levels of PGE₂ were produced by Mφ infected with H37Ra, which increased over time and could be correlated with the MOI (Fig. 2, A and B). Infection with H37Ra also significantly induced production of the prostanoids PGF_{2α} (Fig. 2 C) and TXA₂ (not depicted). In contrast, the virulent *Mtb* strain H37Rv failed to induce significant prostanoid production (Fig. 2, A–C). To determine whether these findings were not confounded by altered bacterial uptake, survival, or replication, we measured bacterial counts. 72 h after infection, the bacterial burden was similar between Mφ infected with H37Rv and H37Ra (unpublished data).

Thus, virulent *Mtb* suppress COX2 expression in infected Mφ, which leads to a global inhibition of prostanoid production. Although prostanoid synthesis is significantly inhibited by H37Rv, the amount that is produced is likely to be an important counterbalance to LXA₄ because Mφ exposed to 1 nM LXA₄ are highly sensitive to PGE₂ and respond to ~1–2 logs lower PGE₂ concentrations than Mφ not exposed to LXA₄ with a protective response against necrosis (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20080767/DC1>).

PGE₂ suppresses mitochondrial inner membrane perturbation (MPT) and necrosis in Mφ infected with virulent *Mtb*

Virulent H37Rv induce rampant necrosis. In contrast, avirulent H37Ra fail to induce Mφ necrosis (7, 8, 12). The observation

that infection of Mφ with H37Rv induces LXA₄ and inhibits COX2 and prostanoid production, whereas H37Ra infection fails to induce LXA₄ but stimulates COX2 and prostanoid production, led to the hypothesis that H37Rv-induced LXA₄ production may foster Mφ necrosis via inhibition of PGE₂ production. Conversely, H37Ra infection may stimulate PGE₂-mediated inhibition of LXA₄ and prevent Mφ necrosis. By determining the release of the cationic dye DiOC₆(3) from mitochondria, a correlate of Mφ necrosis (41), we first investigated whether prostanoids block induction of H37Rv-induced necrosis. Indeed, increasing micromolar concentrations of PGE₂ significantly decreased cationic dye release from the mitochondria of human Mφ infected with virulent H37Rv (MOI 10) after 24 h (Fig. 3, A–C). These results were dupli-

cated in experiments with mouse Mφ (unpublished data). Addition of exogenous PGE₂ also inhibited necrosis measured by 7-AAD staining of H37Rv-infected Mφ. Specifically, addition of 1 μM PGE₂ to H37Rv-infected Mφ decreased the number of 7-AAD-positive Mφ after 48 h from 29 ± 1 to 17 ± 1% after infection with H37Rv (background 2.2 ± 0.5%; MOI 10:1; P < 0.01; n = 3). Other prostanoids, such as PGD₂, PGF_{2α}, PGI₂, and TXA₂, and leukotriene LTB₄, had little or no effect on prevention of necrosis (Fig. 3 C).

We next confirmed the role of PGE₂ in protection against *Mtb* infection-induced necrosis using a loss-of-function approach. In this study, we reasoned that *Mtb*-induced PGE₂ production is a default pathway for the innate immune response, which may be interrupted in H37Rv infection. Thus, infection

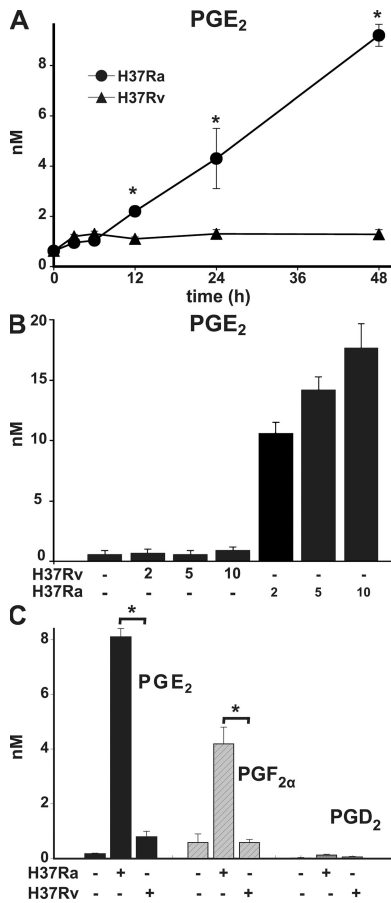


Figure 2. Prostanoid production of Mφ infected with *Mtb*. (A) PGE₂ production of human Mφ 0–48 h after infection with H37Ra and H37Rv (MOI 10:1). Differences in PGE₂ concentrations in supernatants from H37Ra- and H37Rv-infected Mφ are statistically significant (*, P < 0.002; n = 3). (B) PGE₂ production by Mφ infected with H37Ra and H37Rv (MOI 2:1–10:1). The differences in PGE₂ production induced by H37Ra in comparison to H37Rv are statistically significant at all MOIs (*, P < 0.01; n = 3). (C) Quantification of PGE₂, PGF_{2α}, and PGD₂ in 48-h supernatants from human Mφ (5 × 10⁶ /ml) infected with H37Ra and H37Rv (MOI 10:1; *, P < 0.002; n = 4). Data are presented as means ± SE. In all studies, n represents the number of independent experiments.

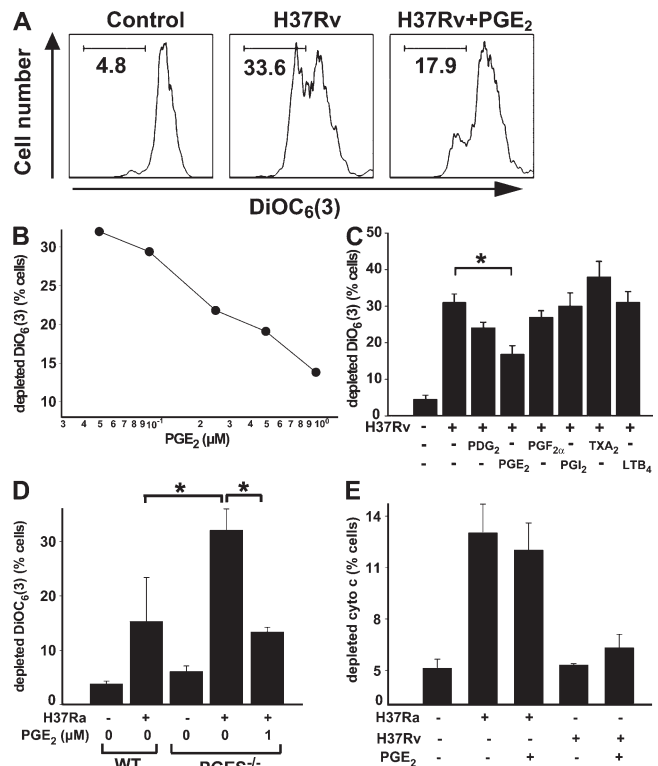


Figure 3. Effect of exogenous prostanoids produced by *Mtb*-infected Mφ on H37Rv-induced mitochondrial cationic dye release. (A) PGE₂ blocks DiOC₆(3) release from mitochondria of human Mφ infected with H37Rv (MOI 5:1; 48 h). A fluorescence-activated cell sorter diagram is shown. (B) Down-regulation of cationic dye release by increasing concentrations of PGE₂ (2–10 μM) is statistically significant at all PGE₂ concentrations (P < 0.01; n = 6). (C) Effect of 1 μM of various prostanoids on H37Rv-induced mitochondrial DiOC₆(3) release of Mφ. Only PGE₂ inhibition of cationic dye release from the mitochondria is statistically significant (*, P < 0.007; n = 3). PGF_{2α} has borderline activity. The concentration of LTB₄ is 0.1 μM. (D) H37Ra (MOI 5:1)-induced DiOC₆(3) release from mitochondria of WT and PGES^{-/-} mouse Mφ infected in the absence and presence of 1 μM PGE₂. At 48 h, cationic dye release from the mitochondria was measured (*, P < 0.006; n = 5). (E) 1 μM PGE₂ does not alter H37Ra- and H37Rv-induced cytochrome c release from the mitochondria (not significant; n = 3). Data are presented as means ± SE. In all studies, n represents the number of independent experiments.

of microsomal prostaglandin E synthase (mPGES)^{-/-} Mφ deficient of PGE₂ production with H37Ra should mimic the virulent status in H37Rv infection and stimulate necrosis. As anticipated, PGES^{-/-} Mφ (42) are unable to produce PGE₂ after infection with avirulent H37Ra and show a concomitant phenotypic switch to susceptibility to necrosis after infection (Fig. 3 D). These results demonstrate that PGES activity inhibits *Mtb*-induced necrosis. Addition of exogenous PGE₂ to H37Ra-infected PGES^{-/-} Mφ restored the avirulent phenotype with striking inhibition of necrosis and confirms that PGE₂ is a central mediator in cell death regulation of *Mtb*-infected Mφ (Fig. 3 D).

To ascertain that induction of human and mouse Mφ necrosis by *Mtb* is caused by related mechanisms, we determined the amount of necrosis after infection with the same MOIs of H37Ra or H37Rv. The results show that both human and mouse Mφ have a similar necrosis response after infection (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20080767/DC1>).

Defining the existence of distinct mechanisms that regulate necrosis and apoptosis, and having observed a PGE₂-dependent modulation in the context of *Mtb* infection, we next investigated whether PGE₂ has a direct role in the regulation of Mφ apoptosis. In this study, we assessed PGE₂-stimulated cytochrome *c* release from the mitochondrial intermembrane space (19), a correlate of apoptosis. Interestingly, we find that PGE₂ does not modulate cytochrome *c* release in H37Ra or H37Rv-infected Mφ (Fig. 3 E), nor does it cause cytochrome *c* release in absence of infection (not depicted). Thus, PGE₂ plays no role in the induction of apoptosis.

Necrosis is positively regulated by LXA₄ through inhibition of prostanoid synthesis leading to mitochondrial inner membrane perturbation

In the mouse model of *Mtb* infection, LXA₄ production correlates with reduced resistance against *Mtb* (38). Our data reveal a relationship between LXA₄ production and Mφ necrosis after virulent *Mtb* infection in vitro. These observations collectively suggest a functional contribution for LXA₄ in the induction of necrosis. We reasoned that if LXA₄ production is a mechanism by which virulent H37Rv promote necrosis in infected Mφ, addition of LXA₄ to Mφ infected with nonvirulent H37Ra should confer a virulent phenotype with regard to *Mtb*-induced necrosis. Indeed, LXA₄ significantly enhanced cationic dye release from the mitochondria, a correlate of necrosis, in H37Ra-infected Mφ (Fig. 4 A, left). Importantly, LXA₄ administration in the absence of *Mtb* infection has no effect on Mφ necrosis (Fig. 4 A, left). Moreover, addition of LXA₄ to H37Ra-infected PGES^{-/-} Mφ, which are unable to synthesize PGE₂, does not increase cationic dye release from the mitochondria (Fig. 4 A, right). These results suggest that in *Mtb*-infected Mφ, LXA₄ acts by down-regulating PGE₂ synthesis to induce necrosis.

To investigate whether induction of necrosis by LXA₄ is based on an inhibitory effect of LXA₄ on PGE₂ synthesis, we determined whether LXA₄ blocks synthesis of COX2, thus inhibiting generation of PGE₂. Indeed, 10⁻⁹ M of stable LXA₄

analogue added to H37Ra-infected Mφ dramatically inhibited both COX2 protein and PGE₂ production induced by H37Ra (Fig. 4 B). Silencing of the 5-LO gene (Fig. 4 C, left) in Mφ infected with the virulent H37Rv inhibited H37Rv-induced LXA₄ production (Fig. 4 C, right) and reduced necrosis (Fig. 4 D), demonstrating that 5-LO activity is required for LXA₄ production by H37Rv-infected Mφ. Finally, inhibition of LXA₄ synthesis in H37Rv-infected Mφ by 5-LO gene silencing led to PGE₂ production (Fig. 4 E). These results not only provide additional proof that LXA₄ suppresses PGE₂ production but demonstrate that the induction of

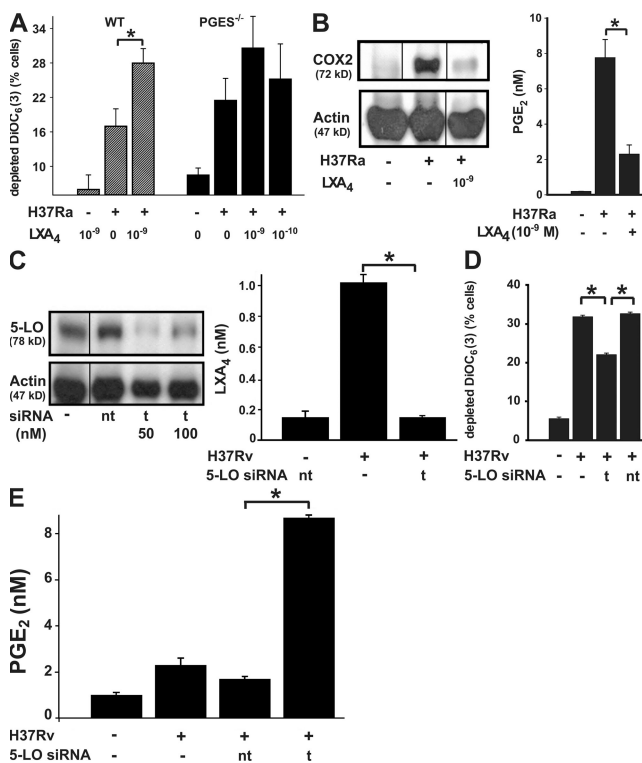


Figure 4. Effect of LXA₄ on *Mtb*-infected Mφ. (A, left) addition of 10⁻⁹ M LXA₄ to H37Ra-infected human Mφ (MOI 10:1) enhances mitochondrial cationic dye release (*, statistically significant difference; *n* = 3; *P* = 0.04). (A, right) LXA₄ by itself is ineffective. Addition of 10⁻⁹ and 10⁻¹⁰ M LXA₄ to PGES^{-/-} mouse Mφ infected with H37Ra (MOI 5:1) does not affect mitochondrial cationic dye release (not significant; *n* = 3). (B, left) Production of COX2 protein by H37Ra (MOI 10:1)-infected Mφ is inhibited by the addition of 10⁻⁹ M LXA₄. Western analysis of Mφ extracts. The actions of LXA₄ were mimicked by its metabolic stable analogue (not depicted). (B, right) PGE₂ production by Mφ (*, *P* < 0.001; *n* = 3). Black lines indicate that intervening lanes have been spliced out. (C and D) Targeted silencing (t) of the 5-LO gene (C, ~70% inhibition at t 50) abrogates production of LXA₄ by H37Rv-infected Mφ compared with nontargeted (nt) silencing (D, left, 50 nM siRNA; *P* = 0.001; *n* = 3) and reduces mitochondrial cationic dye release after H37Rv infection (D, right; nt, nontargeted; *P* < 0.04; *n* = 3). Black lines indicate that intervening lanes have been spliced out. (E) Targeted silencing of the 5-LO gene reconstitutes production of PGE₂ after infection of Mφ with H37Rv, indicating that block of PGE₂ production is caused by the action of LXA₄ (*P* < 0.003; *n* = 3). Data are presented as means ± SE. In all studies, *n* represents the number of independent experiments (*, statistically significant).

LXA₄ by *Mtb* is a potential mechanism that prevents PGE₂ production and leads to cell necrosis.

The prostanoid receptor EP2 is involved in protection of the mitochondrial inner membrane of *Mtb*-infected Mφ by PGE₂

The action of PGE₂ on the Mφ is mediated by four distinct G protein-coupled E prostanoid (EP) receptors, referred to as EP1, EP2, EP3, and EP4 (43), and mice deficient in the four PGE₂ receptors have been generated (44–47). All four receptors are expressed by human Mφ (Fig. 5 A). The EP1 receptor is functionally connected to G_q, a G protein that increases Ca²⁺_i. The EP2 and EP4 receptors activate G proteins that activate adenylate cyclase leading to increased cAMP production. The EP3 receptor inhibits adenylate cyclase via G_i and reduces cAMP levels. To confirm the central role of PGE₂ and to more precisely determine its action via specific EP receptors in protection of the mitochondrial inner membrane from MPT, we determined the effect of exogenous PGE₂ on cationic dye release in WT Mφ and Mφ from homozygote EP1, EP2, EP3, and EP4^{-/-} mice infected with H37Ra and H37Rv. Mφ from WT, EP1, EP3, and EP4^{-/-} mice responded to increasing exogenous PGE₂ concentrations with enhanced inhibition of cationic dye release induced by H37Rv. Cationic dye release from the mitochondria in EP2^{-/-} Mφ infected with H37Rv could not be inhibited even by high concentrations of PGE₂, indicating that EP2 is the major PGE₂ receptor responsible for inhibition of mitochondrial cationic dye release. Identical results were obtained when the cells were infected with H37Ra (Fig. 5 B).

Stimulation of EP2 and EP4 receptors triggers activation of adenylate cyclase (48). EP2 is associated with activation of protein kinase A (PKA) (49–51). In contrast, signaling through EP4 mainly stimulates phosphatidylinositol-3 kinase (PI3K)-mediated processes (49, 52). To confirm that the inhibition of mitochondrial cationic dye release is modulated by stimulation of EP2, we examined the effect of the specific PKA inhibitor KT5720 (53) on PGE₂-dependent inhibition of mitochondrial cationic dye release. We also confirmed lack of involvement of the EP4-stimulated PI3K pathway by testing the effect of the PI3K inhibitor LY294002 on mitochondrial cationic dye release (54). KT5720, at concentrations previously shown to protect cells from apoptosis (10⁻⁷ M [reference 53]), abrogated the protective effect of PGE₂ on Mφ (Fig. 5 C). In agreement with the postulated hypothesis that H37Ra is unable to induce necrosis in infected Mφ because of induction of PGE₂ production, addition of KT5720 to H37Ra-infected Mφ significantly augmented Mφ necrosis (Fig. 5 C). In contrast, LY294002 had no effect (Fig. 5 D). KT5720 and LY294002 alone had no effect on mitochondrial cationic dye release induced by H37Rv. These results are consistent with a mechanism wherein EP2 activation of PKA, rather than EP4 activation of PI3K, mediates the protective effect of PGE₂ on mitochondria of *Mtb*-infected Mφ.

Pulmonary *Mtb* infection in mice is controlled by PGE₂

The findings presented in the previous section indicate that infection with virulent *Mtb* leads to the induction of LXA₄ and suppression of PGE₂, which results in the necrotic death of infected Mφ. We therefore performed in vitro experiments with Mφ from WT and PGES^{-/-} mice. Fig. 6 A shows that after 4 d of H37Rv infection, the bacterial growth was comparable between WT and PGES^{-/-} Mφ. However, 7 d after infection the bacterial burden was significantly higher in PGES^{-/-} Mφ than in WT Mφ. These data suggested that PGE₂ plays a major regulatory role in controlling bacterial growth in Mφ and predicted that an alteration in the balance between PGE₂ and LXA₄ might change the in vivo outcome of infection. Indeed, 5-LO^{-/-} mice are more

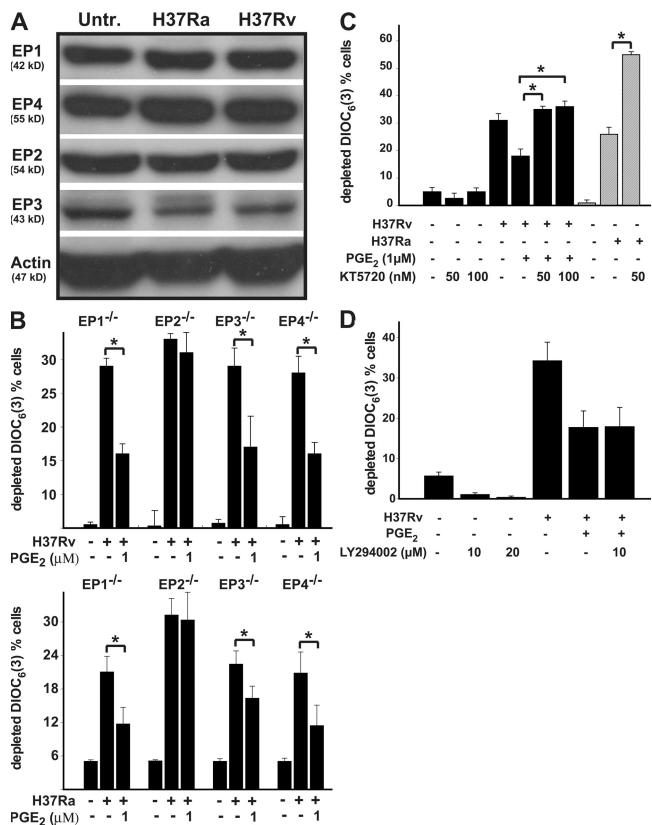


Figure 5. EP2 mediates PGE₂-dependent protection against cationic mitochondrial dye release.

Data are presented as means ± SE. (A) EP1, EP2, EP3, and EP4 are constitutively expressed in human Mφ. Their expression is not increased by either H37Ra or H37Rv infection. (B) EP2^{-/-} Mφ fail to respond to PGE₂ by down-regulating DiOC₆(3) release from the mitochondria infected with H37Rv (top) or with H37Ra (bottom), indicating that EP2 mediates the protective function of PGE₂. Mφ from EP1, EP3, and EP4^{-/-} mice were equally responsive to 1 μM PGE₂ (*, statistically significant; P < 0.01; n = 5). (C) The cAMP-dependent PKA inhibitor KT5720 abrogates inhibition of mitochondrial cationic dye release by PGE₂ (black columns; P < 0.01; n = 3). Addition of KT5720 to H37Ra-infected (MOI 10:1) Mφ enhanced Mφ necrosis (gray columns; *, P < 0.01; n = 3). (D) The PI3K inhibitor LY294002 does not abrogate inhibition of mitochondrial cationic dye release by PGE₂ (not significant; n = 3). In all studies, n represents the number of independent experiments.

resistant to infection (38). Similarly, we would predict that in the absence of PGES, mice are more susceptible to infection. WT and $PGES^{-/-}$ mice were infected by the aerosol route with 100 virulent *Mtb* per lung. Similar numbers of bacteria were found in the lungs of both WT and $PGES^{-/-}$ mice on day 1 after infection ($n = 4/\text{group}$). The bacterial burden in WT and $PGES^{-/-}$ mice was not different 2 wk after infection (unpublished data). However, after 5 wk WT mice controlled the infection more efficiently compared with the $PGES^{-/-}$ mice, whose lungs contained more bacteria ($\Delta \log_{10} = 0.97$; $P = 0.0079$; Fig. 6). This difference was not observed in the spleen (unpublished data). To assess whether the balance between PGE_2 and LXA_4 is changed during the course of a mycobacterial infection in vivo, we next measured the levels of PGE_2 and LXA_4 in sera from WT mice infected with either virulent Erdmann strain or avirulent H37Ra. As it was observed in vitro, virulent bacteria induced remarkably more LXA_4 after 7 d of infection, whereas avirulent H37Ra induced more PGE_2 . This indicates that PGE_2 significantly contributes in vivo to protective responses against mycobacterial infection in the lung.

DISCUSSION

The central finding of this study is that in the local environment of an *Mtb* infection, $M\phi$ infected with virulent *Mtb* preferentially synthesize LXA_4 and little, if any, PGE_2 . In contrast, $M\phi$ infected with avirulent H37Ra produce mainly prostanoids, including PGE_2 , and only small amounts of LXA_4 .

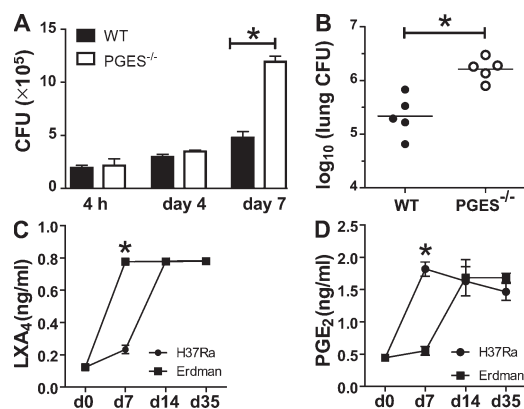


Figure 6. Mycobacterial burden of $PGES^{-/-}$ and WT $M\phi$ in vitro and in vivo. (A) Mycobacterial burden after 4 h (inoculum), 4 d, and 7 d of $PGES^{-/-}$ and WT $M\phi$ in vitro infected with H37Rv (MOI 10:1). The difference in the bacterial burden was significant at 4 and 7 d after infection (*, $P < 0.03$). (B) 5 wk after aerosol infection, CFUs were determined by plating of homogenized lung tissue as indicated on the ordinate. The difference in mycobacterial burden in the lungs of $PGES^{-/-}$ versus WT mice is statistically significant (*, $P = 0.002$; five mice per group). (C and D) Induction of LXA_4 and PGE_2 production during the course of mycobacterial infection. WT mice were infected by aerosol exposure with virulent (Erdmann) or avirulent (H37Ra). LXA_4 (C) and PGE_2 (D) measured by ELISA in the sera at 7, 14, and 35 d after infection (*, $P < 0.01$; three mice per time points). These results are representative of two independent experiments. The error bars represent SE.

Our results demonstrate hitherto unappreciated disparate roles for LXA_4 and PGE_2 in the regulation of induction of host $M\phi$ necrosis, a cell death leading to cytolysis. $M\phi$ infected with virulent *Mtb* that produce LXA_4 and reduced amounts of PGE_2 undergo necrosis. In contrast, prevention of necrosis and concomitant induction of $M\phi$ apoptosis is associated with increased PGE_2 production. Thus, the induction of LXA_4 and the resulting inhibition of PGE_2 production contribute to the virulence of *Mtb* and enhance its capacity to evade killing by the innate immune system. Indeed, the failure of $PGES^{-/-}$ $M\phi$ to control H37Rv replication in vitro and the observation that $PGES^{-/-}$ mice have a 10-fold higher *Mtb* burden in their lungs compared with WT mice demonstrate the protective effect of PGE_2 against virulent *Mtb*. Preferential synthesis of either LXA_4 or of prostanoids is a critical branch point for the innate antimycobacterial response of the infected $M\phi$ (Fig. 7).

Lipoxins are best described as potent negative regulators of acute inflammatory processes (55), in part via inhibition of DC mobilization, inhibition of IL12 production (56), and PMN-induced inflammation in vivo. LXA_4 activities are multifold and include inhibition of PMN entry to sites of inflammation, reduction of vascular permeability, promotion of monocyte infiltration, and ingestion of apoptotic cells, perhaps including infected $M\phi$ by phagocytes (39). Moreover, lipid mediators that resolve inflammatory events, including LXA_4 , stimulate phagocytosis of zymosan and of microbial organisms leading to de novo infection of phagocytes (56). The results suggest that the action of LXA_4 in reducing prostanoid production as part of the inflammation-resolving program of LXA_4 is exploited by *Mtb* in the local environment. Thus, although temporally regulated dampening of the inflammatory response is generally highly beneficial to the host, our studies uncover a novel scenario wherein LXA_4 -driven reduction of “proinflammatory” PGE_2 synthesis functionally acts as an efficient mechanism used by pathogenic *Mtb*. More specifically, LXA_4 counteracts the

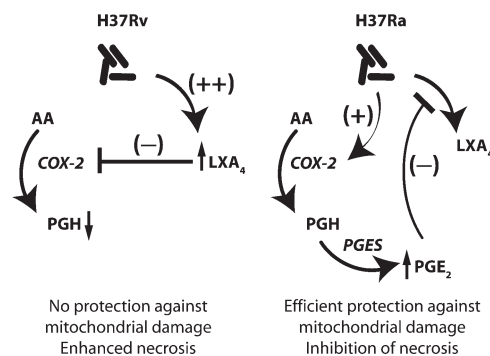


Figure 7. Infection of $M\phi$ with the virulent H37Rv predominantly induces LXA_4 production and block of COX2 and PGE_2 production, which might lead to necrotic cell lysis and spread of the infection.

In contrast, $M\phi$ infected with avirulent H37Ra produce larger amounts of PGE_2 which blocks LXA_4 production and supports $M\phi$ apoptosis and containment of the *Mtb*. Predominant production of either PGE_2 or LXA_4 by $M\phi$ infected with H37Ra or H37Rv, respectively, is supported by the fact that PGE_2 inhibits LXA_4 production and vice versa.

protective effects of PGE₂ by inhibiting COX2 expression, thereby causing necrosis in *Mtb*-infected Mφ. Interestingly, LXA₄ on its own does not induce necrosis, indicating that the main function of LXA₄ is down-regulation of prostanoid production followed by mitochondrial inner membrane destabilization. These results are consistent with studies demonstrating that mycobacterial burden is significantly reduced in 5-LO^{-/-} mice in comparison to WT mice (38) and suggest that LXA₄ negatively regulates antimycobacterial defense responses. It should be noted that the concentrations of LXA₄ produced are not sufficient to affect inflammatory events in other experimental systems, a feature which is almost certain to be beneficial to the *Mtb* pathogen. Consequently, *Mtb* is able to escape from the necrotic Mφ without adverse effects and is able to infect newly recruited Mφ.

The findings in this paper argue that inhibition of PGE₂ comprises a mechanism specific for virulent *Mtb*, a distinctly noninflammatory role for this prostanoid in the regulated response to this pathogen. Prostanoids, including PGE₂, are the final product of the PGH synthases COX1 and 2. PGE₂ has been extensively studied and is well known for its role in mediating cardinal features of inflammation, including pain, vasodilatation essential for the control of blood flow, and leukocyte diapedesis leading to edema and fever (34). We now show that the prostanoid PGE₂ protects against MPT and necrosis of Mφ infected with virulent *Mtb*. Our data are consistent with recently published findings in several other experimental systems that document an inhibitory role of PGE₂ in cell death. This mechanism is clearly complex and can be mediated in those studies either by the EP2 receptor (57, 58) or the EP4 receptor (59). Although we definitively confirm the role of PGE₂ and more precisely define its mechanistic activity by identifying EP2 as the crucial PGE₂ receptor, these findings beg for further dissection of this important signaling pathway leading to increased Mφ defense against *Mtb*.

The mechanisms by which PGE₂ stimulates induction of apoptosis of *Mtb*-infected Mφ is not understood. Although PGE₂ stimulation of the EP4 receptor triggers activation of PI3K and of Akt (48), blockade of mitochondrial damage by PGE₂ critical for prevention of necrosis is mediated through the EP2 receptor. The EP2 subtype couples to G protein α_s and triggers intracellular cAMP formation. cAMP activates the PKA pathway (45), which inhibits death in several cell types (58). PKA activation was found to block cell death by phosphorylation of the proapoptotic protein Bad (49, 50, 54), causing its inactivation, and also increases the expression of Bcl-2 involving the CREB (cAMP responsive element-binding) protein and inhibition of cell death (59). At present, we do not know which of these mechanisms is involved in the mitochondrial inner membrane stabilization by PGE₂ and in the inhibition of LXA₄ production.

The innate immune system is the first line of defense against invading microbial pathogens and viruses (60). Apoptosis characterized by intact plasma membranes, formation of apoptotic bodies and DNA fragmentation is an important component of the innate immune defense against *Mtb* and

is the predominant death modality of the host Mφ infected with attenuated *Mtb* including H37Ra and secA2 mutants (13). Apoptotic bodies are thought to provide a containment vessel for the bacilli as well as an efficient means for pathogen killing (15). Moreover, apoptotic bodies express specific cell surface receptors (61) that promote uptake and subsequent antigen presentation to DC (14). Virulent *Mtb* subvert apoptosis and induce plasma membrane lysis (necrosis), leading to dissipation of the pathogens and spread of the infection.

Thus, the fate of the infected Mφ with regard to cell death modality is a critical determinant in an effective host defense response to *Mtb* infection. Our studies identify a novel mechanism wherein *Mtb* stimulates a pathway involved in the local resolution of inflammation to subvert defense mechanisms against *Mtb*. To our knowledge, this is the first example of a pathway important in the resolution of inflammation that is targeted by a pathogen to counteract the innate immune system. Although it is tempting to advocate therapeutic targets of the lipoxin pathway for *Mtb* infection, further studies are required both to understand the mechanisms by which LXA₄ production is activated via virulent *Mtb* and to determine the consequences of blocking the LXA₄ production in the course of an infection of Mφ with *Mtb* for the host.

MATERIALS AND METHODS

Materials. LY294002, KT5720, and propidium iodide (PI) were obtained from Sigma-Aldrich. DiOC₆(3) (3,3'-dihexyloxy carbocyanine iodide), rhodamine-2 AM, goat anti-mouse IgG₁, and anti-mouse COX1 antibodies were obtained from Invitrogen. PGE₂, PGF_{2α}, PGD₂, PGI₂, LTB₄, TXB₂, and LXA₄, sheep anti-15-LO antibody, and mouse anti-COX2 antibody were obtained from Cayman Chemical. 15-epi-16-phenoxy-paraffluoro-LXA₄-methyl ester was a gift from Bayer-Schering Pharma AG. Anti-β-actin mAb was obtained from Thermo Fisher Scientific. Mouse anti-5-LO antibody, mouse IgG₁, and mouse anti-cytochrome *c* mAb (7H8.2C12.6H2.B4) were obtained from BD. IMDM, RPMI-1640, Opti-MEM I reduced serum medium, Oligofectamine, Hepes, and DTT were obtained from Invitrogen. Rabbit anti-annexin-1 polyclonal antibody, goat anti-rabbit IgG FITC conjugate, and HRP protein A were obtained from Invitrogen. Anti-phosphatidyl-serine mouse mAb (clone1H6) and rabbit IgG were obtained from Millipore.

Mice. 6–10-wk-old C57BL/6 mice were obtained from Jackson Immuno-Research Laboratories. mPGES-1^{-/-} mice (N5 backcross onto the C57BL/6 background, 45) and EP1 (Ep1^{-/-}; reference 47), EP2^{-/-} (48), EP3^{-/-} (47), and EP4^{-/-} mice (51) (*n* > 10 on C57BL/6 background, provided by B. Koller, University of North Carolina, Chapel Hill, NC) were bred locally. All procedures were approved by the Dana-Farber Cancer Institute Institutional Animal Care and Use Committee.

Bacteria. The virulent *Mtb* strain H37Rv and the attenuated H37Ra (American Type Culture Collection), prepared as described previously (8), were used in the in vitro experiments. The strains were grown in Middlebrook 7H9 broth (BD) with BBL Middlebrook OADC Enrichment (BD) and 0.05% Tween 80 (BD) and resuspended in 7H9 broth at 5 × 10⁷ CFU/ml. Aggregation was prevented by sonication for 10 s. The bacteria were allowed to settle for 10 min. Bacteria in Mφ were quantified by lysis of the cells with 0.2% SDS in PBS. After neutralization of SDS with 50% FCS, 100 μl of cell lysates of triplicate cultures were serially diluted 10-fold, plated on 7H10 agar plates (Remel), and colonies were counted after 21 d. Alternatively, the pooled cell lysates were inoculated into triplicate Bactec 12B vials. The number of bacteria was determined with the Bactec model 460 TB system (BD).

Cells and culture. Mononuclear cells from leukopacks of healthy donors or from mouse spleens (7) were plated for FACS analysis at 4×10^5 cells/ml/well in 12-well cluster plates (Corning) and for PI staining and transfection with small interfering RNA (siRNA) at 5×10^5 cells/ml/well in 12-well cluster plates. Human M ϕ were cultured for 7 d in IMDM with 10% human AB serum (Gemini Bio-Products) and mouse spleen M ϕ for 8–10 d in RPMI 1640 (Invitrogen) with 10% fetal bovine serum (Gemini Bio-Products), 1% Hepes, 1% penicillin/streptomycin, and 0.1% β -mercaptoethanol.

In vitro infection of M ϕ . CD11b⁺ cells were purified from thioglycollate (3%)-elicited peritoneal M ϕ harvested from WT and PGES^{-/-} mice by MACS column purification. The purity of the CD11b⁺ M ϕ was 90%. CD11b⁺ M ϕ (10^5 /well) were allowed to adhere in a 96-well culture plate for 24 h. Adherent cells were infected with virulent H37Rv (MOI 10: 1) for varying time periods. At different time points, cells were washed extensively with PBS and lysed in H₂O for 5 min, and mycobacterial growth was evaluated by enumeration of the bacilli for 21 d by plating the cell lysates on Midlebrook 7H10 agar plates and incubating at 37°C.

Aerosol infection of mice. C57BL/6 and mPGES-1^{-/-} mice were infected with virulent *Mtb* (Erdman strain) via the aerosol route using a nose-only exposure unit (Intox Products) and received ~100 CFU/mouse (3, 15, 16). After 5 wk, mice were killed by CO₂ inhalation and the left lung was aseptically removed and individually homogenized in 0.9% NaCl-0.02% Tween 80 with a Mini-Bead Beater 8 (BioSpec Products, Inc.). Viable bacteria were enumerated by plating 10-fold serial dilutions of organ homogenates onto 7H11 agar plates (Remel). Colonies were counted after 3 wk of incubation at 37°C.

LC-MS-MS and ELISA. PGE₂, PGF_{2 α} , PGD₂, and LXA₄ concentrations in cell supernatants were determined using ELISA kits (Oxford Biomedical Research) according to the recommendations of the manufacturer. The TXA₂ concentration was determined with the TXB₂ express EIA kit (Cayman Chemical). 50 μ l of culture media from infected M ϕ was added to wells in duplicate and absorbance was determined using a microplate reader. The lipid concentration was determined using standard curves. LXA₄ and PGE₂ identity were confirmed using LC-MS-MS diagnostic ions and their physical properties. For LC-MS-MS, an LCQ ion trap mass spectrometer (Finnigan Corp.) with an electrospray ionization probe was used (62).

Assessment of MPT in M ϕ . MPT was assessed in M ϕ by measuring retention of the cationic dye DiOC₆(3) within the mitochondria (41). Cells were loaded with 3 nM DiOC₆(3) in IMDM for 20 min at 37°C. After washing, 20 μ g digitonin was added per milliliter and the M ϕ were incubated at 37°C for 20 min and fixed with 4% paraformaldehyde for 20 min at room temperature. Cells were dislodged with a rubber policeman, washed with PBS, and resuspended in PBS with 1% BSA. Flow cytometry was performed under FL-1 conditions using a BD FACSort flow cytometer (BD).

Western blotting. After incubation with *Mtb* (MOI 10:1), cells were harvested and lysed with SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% wt/vol SDS, 10% glycerol, 50 mM DTT, 0.01% wt/vol bromophenol blue). The cell lysates were sonicated for 10 s, centrifuged at 10,000 *g* for 10 min, and dissolved in 15% SDS by using β -actin as a loading control. Mouse anti-COX2 antibody (1:1 dilution), anti- β -actin (1:2,000 dilution; Thermo Fisher Scientific) antibody, mouse anti-5-LO antibody (1:250 dilution; BD) and ovine anti-15-LO antibody (1:250 dilution; Cayman Chemical) were used to detect 5-LO and 15-LO. Rabbit anti-EP1, -EP2, -EP3, and -EP4 receptor antibodies (1:200; Cayman Chemical) were used to detect EP1, EP2, EP3, and EP4 receptor protein.

RT-PCR. Total RNA was extracted as specified in cells-to-cDNA II kit (Ambion). In brief, 5×10^5 mononuclear cells/ml/well in 12-well cluster plates were dislodged and lysed with 100 μ l of ice-cold cell lysis II buffer and then 2 μ l DNase I was added. Primer sequences used for COX2 were

TTCAAATGAGATTGTGGGAAAATTGCT and AGATCATCTCTG-CCTGAGTATCTT. Primer sequences for GAPDH were ACCACAG-TCCATGCCATCAC and TCCACCACCCTGTTGCTGTA.

PI staining. Adherent mPGES^{-/-} spleen M ϕ adherent were stained with 2.5 μ g/ml PI in RPMI containing 10% FBS at 4°C for 10 min and washed with ice-cold PBS and H₂O. Dried and mounted coverslips were examined using a fluorescence microscope and photographed with a digital camera (DFC300; Leica).

siRNA transfection. The 5-LO siRNA target sequence (AAATGCCA-CAAGGATTTACCC) targeted against NM_000698 and a scrambled control siRNA sequence (GCCCTCTATCGAATAAGACAA) designed with siRNA target finder (Ambion) (63) was used. The siRNAs were synthesized from DNA templates with the Silencer siRNA construction kit (Ambion) according to the manufacturer's instructions. Cells were cultured in IMDM with 10% human AB serum, and the medium was changed 1 d before transfection. All siRNAs were used at a final concentration of 50 nM by diluting with Opti-MEM I reduced serum medium. To oligofectamine (1:200 dilution; Invitrogen), fresh IMDM containing 30% human AB serum (Gemini Bio-Products) was added to bring the serum concentration to 10%. After 72 h of transfection at 37°C, the cells were infected with *Mtb*. To examine the effect of siRNA transfection, cells were harvested and analyzed using Northern or Western blotting.

Statistics. Results are expressed as mean \pm SEM. The data were analyzed by using Excel Statistical Software (Microsoft) using the Student's *t* test for normally distributed data with equal variances. CFU were log₁₀ transformed and analyzed using the Mann Whitney nonparametric *t* test. A *p*-value <0.05 was considered statistically significant.

Online supplemental material. Fig. S1 shows sensitization of human M ϕ with 1 nM LXA₄, 1 h before infection with H37Ra (MOI 10:1) to decreasing concentrations of PGE₂. Fig. S2 shows FACS analysis of necrotic cells (7-ADD) in human and murine M ϕ cultures infected with MOI 10:1 and 20:1 H37Rv and H37Ra for 72 h.

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