

An Important Role of Prostanoid Receptor EP2 in Host Resistance to *Mycobacterium tuberculosis* Infection in Mice

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(See the editorial commentary by Kaushal, on pages 1803–5.)

***Mycobacterium tuberculosis*, the causative agent of tuberculosis, resides and replicates within susceptible hosts by inhibiting host antimicrobial mechanisms. Prostaglandin E₂ (PGE₂), produced by *M. tuberculosis*-infected macrophages, exerts a variety of immunomodulatory functions via 4 receptors (EP1–EP4), each mediating distinct PGE₂ functions. Here, we show that *M. tuberculosis* infection selectively upregulates EP2 messenger RNA expression in CD4⁺ T cells. We found that EP2 deficiency in mice increases susceptibility to *M. tuberculosis* infection, which correlated with reduced antigen-specific T-cell responses and increased levels of CD4⁺CD25⁺Foxp3⁺ T-regulatory cells. These findings have revealed an important role for EP2 in host immune defense against tuberculosis. As a G protein-coupled receptor, EP2 could serve as a target for immunotherapy of tuberculosis.**

Tuberculosis continues to be a major health problem worldwide, affecting >9 million people and killing >1 million people annually [1]. *Mycobacterium tuberculosis*, the causative agent of tuberculosis, modulates host immunity to delay immune responses and to facilitate chronic infection. Delayed generation of antigen-specific T cells, which depends heavily on the multiplicity of infection, and reduced homing of infected antigen-presenting dendritic cells (DCs) to draining lymph nodes are common features of *M. tuberculosis* infection in both humans and mice [2].

Virulent *M. tuberculosis* interferes with host eicosanoid metabolism as a survival strategy. Eicosanoid

bioactive molecules, such as prostaglandin E₂ (PGE₂), are produced by antigen-presenting cells and play an important role in the outcome of polarization of immune responses [3]. *Mycobacterium tuberculosis* modulates host innate immune responses by subverting PGE₂-mediated effects on cytokine and antibody production, phagocytosis, and cell multiplication [4, 5]. Interestingly, PGE₂ exhibits both stimulatory and inhibitory effects on DCs depending on its site of activity and the type of receptor engaged [3]. Therefore, manipulation of PGE₂ responses may serve as an effective strategy for immunomodulation of *M. tuberculosis* infection.

PGE₂ stimulates DCs and macrophages and promotes DC migration from tissue to lymph nodes [3]. Consistent with these findings, some reports have suggested that PGE₂ induces inflammatory responses in lungs, promoting vasodilation, vascular permeability, and bronchodilation [4]. However, following migration of DCs to lymph nodes, PGE₂ can suppress the maturation and antigen-presenting functions of these cells [3]. Furthermore, it has been suggested that PGE₂ elicits anti-inflammatory responses and induces

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immune suppression in part by decreasing T-cell proliferation, interleukin 2 production, and interleukin 2 receptor expression [3]. In addition, PGE₂ inhibits activation-induced cell death in effector T cells by preventing CD95L upregulation in response to T-cell receptor (TCR) engagement [6]. We have recently shown that PGE₂ is particularly critical for preventing activation-induced cell death in T helper 2 (Th2) cells [7]. Taken together, these studies have provided evidence that PGE₂ plays a major role in modulating adaptive immune responses.

PGE₂ has four distinct receptors (EP1–EP4) that vary widely in expression and downstream intracellular signaling pathways, leading to an increase or attenuation of inflammatory and protective immune responses [3]. It is well documented that PGE₂ suppresses DC functions through an EP2/EP4-dependent mechanism [3], whereas it acts via the EP2 receptor on T regulatory cells (Tregs) to inhibit their expansion [8]. Consistent with these findings, signaling through EP2 exhibited a potent antagonism of transforming growth factor beta (Tgf-β)-mediated activities in normal and malignant mammary epithelial cells [9].

Previous studies have shown that PGE₂ levels increase during the course of *M. tuberculosis* infection and that the outcome of adaptive immune responses against this microorganism is influenced by PGE₂ levels and by the stage of disease progression [5]. The relatively low levels of PGE₂ that are present during the early stages of *M. tuberculosis* infection counteract bacterial growth in a manner dependent on inducible nitric oxide synthase, whereas the high PGE₂ levels that are present during late stages of infection permit disease progression [5]. Consequently, inhibition of PGE₂ synthesis during the early phase of *M. tuberculosis* infection enhances bacterial burden, with a concomitant increase in inflammation and expression of tumor necrosis factor α, interleukin 1α, and interferon γ (IFN-γ) and a nearly complete abrogation of inducible nitric oxide synthase expression [5].

Infection with virulent *M. tuberculosis* induces lipoxin A4 (LXA4) production and suppression of PGE₂ synthesis in macrophages, which leads to necrosis of infected macrophages and increased disease [4]. Consistent with these findings, macrophages from PGE₂ synthase-deficient (PGES^{-/-}) mice fail to control virulent *M. tuberculosis* replication in vitro [4]. Furthermore, PGES^{-/-} animals exhibit a 10-fold increase in *M. tuberculosis* burden in their lungs, as compared with wild-type (WT) mice [4]. It has been suggested that PGE₂ protects the mitochondrial inner membrane of *M. tuberculosis*-infected macrophages, preventing their necrosis, in a manner that involves the EP2 receptor [4]. In this way, it is thought that PGE₂ favors apoptosis of infected macrophages and promotes cross-presentation of mycobacterial antigens by DCs to prime T-cell responses.

Although it is widely recognized that PGE₂ influences the activity of Tregs and effector T cells, few studies have

evaluated the direct effects of PGE₂ on T cells during the course of *M. tuberculosis* infection. Studies by Garg et al have shown that human Tregs expand in response to *M. tuberculosis* infection in a manner that is dependent on PGE₂. However, these studies involved ex vivo treatment of isolated CD4⁺ T cells from healthy tuberculin-positive subjects with *M. tuberculosis* antigens [10]. Thus, the effects of PGE₂ receptor signaling in T cells on the development of protective immune responses during *M. tuberculosis* infection remain to be explored.

Here, we report that *M. tuberculosis* infection upregulates messenger RNA (mRNA) expression of EP2. We found that EP2-deficient (EP2^{-/-}) mice exhibit increased susceptibility to *M. tuberculosis* infection, with a concomitant increase in CD4⁺CD25⁺Foxp3⁺ Tregs. Thus, our findings demonstrate that the EP2 receptor plays an important role in the outcome of *M. tuberculosis* infection by altering the production of immunosuppressive cytokines and Tregs. Our findings also identify EP2 as a possible new small-molecule target for immunomodulation during *M. tuberculosis* infection.

METHODS

Ethics Statement

All animal experiments were performed in accordance with guidelines developed and approved by the Institutional Animal Ethics Committee meeting held on 22 November 2007 at the International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi, India (approval ID: ICGEB/IAEC/IMM-13/2007) and by Department of Biotechnology guidelines, Government of India.

Mice

C57BL/6 mice (aged 6–8 weeks) were initially purchased from the Jackson Laboratories. Prostaglandin receptor subtype EP2^{-/-} mice, originally generated by Breyer and colleagues [11] were obtained from the Jackson Laboratories. All animals were subsequently bred and maintained in our specific pathogen-free animal facility of the ICGEB. Infected mice were housed under barrier conditions in a biosafety level 3 laboratory.

Antibodies and Reagents

Fluorophore-tagged antibodies against mouse CD4, CD25, Foxp3, interleukin 17A (IL-17A), interleukin 4 (IL-4), and isotype controls were purchased from BD Biosciences. Luminex multiplex cytokine detection kits were purchased from Bio-Rad or Millipore. CD4⁺ T-cell-specific microbeads, magnetic-activated cell sorting (MACS) columns, and nylon-mesh filters were obtained from Miltenyi Biotech. Fine chemicals, primers, and antibiotics were procured from Sigma-Aldrich. Tritiated thymidine was purchased from PerkinElmer.

***Mycobacterium tuberculosis* Aerosol Infection and Colony-Forming Unit Estimation**

Mycobacterium tuberculosis strain H37Rv (American Type Culture Collection 27294) was grown in Middlebrook 7H9 medium (Difco) supplemented with 0.1% Tween 80 (Sigma-Aldrich), 0.2% glycerol, and 10% v/v albumin, dextrose, and catalase (Difco) as recommended by the manufacturer. Bacterial cultures were grown up to midlog phase (optical density 600, approximately 0.6). Aliquots of bacterial cultures were prepared in 20% glycerol and Middlebrook 7H9 medium and preserved at -80°C . Colony-forming units (CFUs) per milliliter of the stocks were determined by plating on Middlebrook 7H11 plates supplemented with 10% oleic acid, albumin, dextrose, and catalase (Difco). These stocks were used for all subsequent aerosol infections in mice, as per the protocol described elsewhere [12].

T-Cell Proliferation Assay

Splenocytes were isolated from uninfected and infected mice, and red blood cells present in the single-cell suspension of splenocytes were lysed with hypotonic red blood cell lysis buffer. Total splenocytes were used for mixed lymphocyte reactions in the presence of mycobacterial complete soluble antigen. Cells were plated at 0.5×10^6 cells/well in a 96-well plate, cultured for 72 hours, and then pulsed with tritiated thymidine ($1 \mu\text{Ci/well}$) for the last 14 hours of incubation before measuring incorporation of tritiated thymidine using cell harvester and liquid scintillation counter (Wallac Trilux, Perkin-Elmer).

Detection of Cytokines

Splenocytes from infected and uninfected mice were stimulated in the presence of complete soluble antigen at different concentrations and cultured for 48 hours. Culture supernatants were used to detect T-cell cytokines in a Luminex microbead-based multiplexed assay (Qiagen Luminex Liquichip) using commercially available kits according to manufacturer's protocol (BioPlex, Bio-Rad).

Flow Cytometry: Surface and Intracellular Staining

Splenocytes were isolated from uninfected and infected mice. Red blood cells present in the single-cell suspension of splenocytes were lysed with hypotonic red blood cell lysis buffer. Cells were counted and 1×10^6 cells were stained with fluorescently conjugated monoclonal antibodies for surface markers.

Intracellular staining of splenocytes was done using the fluorescently labeled antibodies for anti-IL-4 (clone:11B11), anti-IL-17 (clone: 17B7)-phycoerythrin (PE), and Foxp3 (clone: FJK-16) as described elsewhere [12]. Fluorescence intensity was measured by flow cytometry (FACS CantoTM II, BD Biosciences) and data analysis was performed by FlowJo (Tree Star).

Quantitative Real-Time Polymerase Chain Reaction

CD4^+ T cells were isolated from uninfected and infected mice by positive selection using MACS. Total RNA isolation was performed using RNAeasy isolation kit (Qiagen) according to manufacturer's instructions. After DNase treatment, complementary DNA (cDNA) was synthesized using iScript cDNA synthesis kit (Bio-Rad). Real-time polymerase chain reaction (PCR) analysis was performed using Bio-Rad CFX96 real-time thermal cycler and IQBio-Rad SYBR green master mix (Bio-Rad) for *Gata-3*, *Foxp3*, and *Tgf- β* expression, respectively. Relative expression level of mRNAs was normalized to that of internal control *Gapdh* by using $2^{-\Delta\Delta\text{Ct}}$ cycle threshold method. Primer sequences used were as follows: *Gata-3* (forward): 5'-TTATCAAGCCCAAGCGAAGG-3' and *Gata-3* (reverse): 5'-CATTAGCGTTCCTCCTCCAGAG-3'; *Foxp3* (forward): 5'-GGCCCTTCTCCAGGACAGA-3' and *Foxp3* (reverse): 5'-GCTGATCATGGCTGGGTTGT-3'; *Tgf- β* (forward): 5'-ACCATGCCAACTTCTGTCTG-3' and *Tgf- β* (reverse): 5'-CGGGTTGTGTTGGTTGTAGA-3'; and *Gapdh* (forward): 5'-TCTGACCACAGTGAGGAATGTCCAC-3' and *Gapdh* (reverse): 5'-TTGATGGCAACAATCTCCAC-3'.

Immunohistochemistry

Lungs were infused and fixed in 10% paraformaldehyde in phosphate-buffered saline, paraffin embedded, cut, and stained with hematoxylin and eosin according to standard protocols. Lung tissue sections were stained for immunohistochemistry analysis of Tgf- β . Paraffin-embedded blocks were cut in sections of 5–7 μm . The sections were prepared for

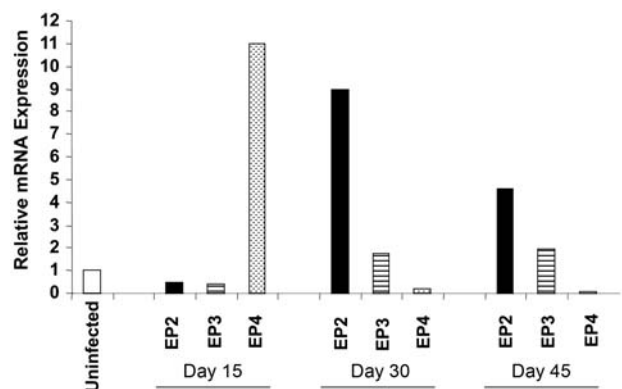


Figure 1. Regulation of EP2 messenger RNA (mRNA) expression in CD4^+ T cells by *Mycobacterium tuberculosis* and its role in tuberculosis pathogenesis in mice. Mice were challenged with a low dose of *M. tuberculosis* by the aerosol route (110 bacilli). CD4^+ T cells were purified from splenocytes of uninfected and infected C57BL/6 (wild-type) mice, and EP receptor (EP2, EP3, EP4) expression levels were measured in CD4^+ T cells at the indicated time points after infection by real-time polymerase chain reaction. Data are representative of 2 independent experiments (3 mice per experiment).

immunohistochemistry as described elsewhere [13]. Primary antibody for mouse Tgf- β (Santa Cruz Biotechnology) was used at 1:100 dilution.

Statistical Analysis

Student unpaired *t* test was performed to compare the statistical significance between various groups. *P* values were considered significant if $P \leq .05$. Standard deviation of the mean or standard error of the mean is shown in the figures. GraphPad Prism 5.0 software was used for statistical analyses.

RESULTS

Expression of EP Receptors During *M. tuberculosis* Infection

To explore the role of EP receptors during *M. tuberculosis* infection, we first evaluated the mRNA expression levels of the 4 EP receptors during disease progression. Wild-type C57BL/6 mice were infected with a low dose of *M. tuberculosis* (110 bacilli), and expression of EP receptors in CD4⁺ T cells isolated from the spleen was evaluated by real-time PCR at different time points after infection. We found that expression of EP2 was significantly upregulated after infection (Figure 1). EP1 was weakly expressed, if any, in the absence or presence of infection (data not shown), and EP3 expression was not significantly altered by infection. Surprisingly, EP4 was highly expressed 15 days after infection, but expression declined thereafter. However, its regulation did not correlate with microbial burden because negligible CFUs were detected in the spleen 15 days after infection (Figure 2). In contrast, EP2 mRNA expression was consistently upregulated later during infection, at days 30 and 45 (Figure 1).

Role of EP2 in Tuberculosis Pathogenesis

EP2 has been shown to play a role in many immunological functions, such as antigen-specific T-cell generation and T-cell polarization, favoring Th2 cell differentiation [14, 15]. It has been well documented that *M. tuberculosis* polarizes Th2-cell responses in susceptible hosts as a survival strategy [16]. Therefore, we next examined whether EP2 plays a role in tuberculosis pathogenesis. We infected EP2^{-/-} mice with a low-dose of *M. tuberculosis* through the aerosol route and determined bacterial load in lungs and spleen at different time points. We found only a few CFUs present in the spleen and lungs of WT and EP2^{-/-} mice at 15 days after infection (Figure 2). In contrast, we detected dramatically increased amounts of CFUs in spleen and lungs of EP2^{-/-} mice compared with WT mice at 30 days after infection (Figure 2). The CFU levels in the lungs but not the spleen of EP2^{-/-} mice remained increased at day 45. These data indicated that EP2^{-/-} mice have a relative increased susceptibility to *M. tuberculosis* infection compared with WT animals.

EP2^{-/-} Animals Exhibit Hyperactive Immune Responses to *M. tuberculosis* Antigens

Because EP2^{-/-} animals exhibited increased susceptibility to *M. tuberculosis* infection, we next examined whether these mice are impaired in mounting antigen-specific proliferative T-cell responses. To test this hypothesis, we harvested splenocytes from infected WT or EP2^{-/-} mice and rechallenged these cells *ex vivo* with *M. tuberculosis* complete soluble antigen. Proliferation was measured by tritiated thymidine incorporation. We observed that splenocytes from EP2^{-/-} mice proliferated at much higher levels than splenocytes from WT animals (Figure 3). This observation indicated that EP2^{-/-}

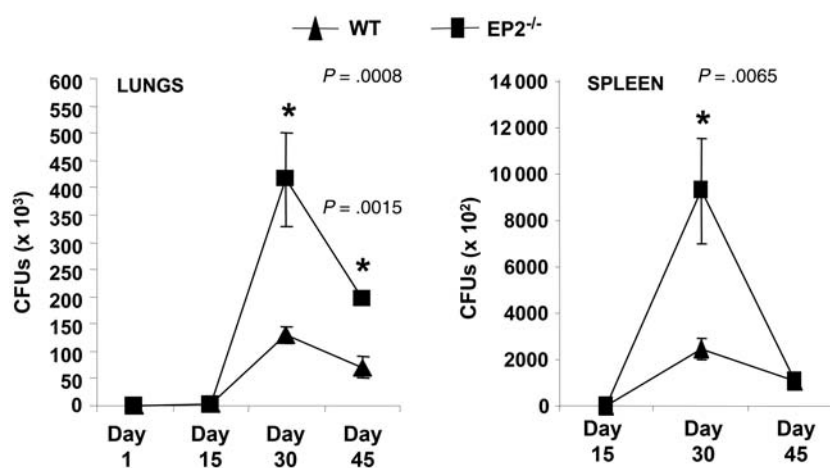


Figure 2. EP2^{-/-} mice have increased bacterial burden following *Mycobacterium tuberculosis* infection. EP2^{-/-} and C57BL/6 (wild-type) mice were challenged with *M. tuberculosis* by the aerosol route (low-dose, 110 bacilli) and colony-forming units (CFUs) were determined in lungs and spleen of infected mice at the indicated time points after infection. Data shown here are cumulative of 3 experiments, representing mean \pm standard deviation values of 8–10 mice per group. Each CFU count was carried out in triplicate (3–5 mice per experiment).

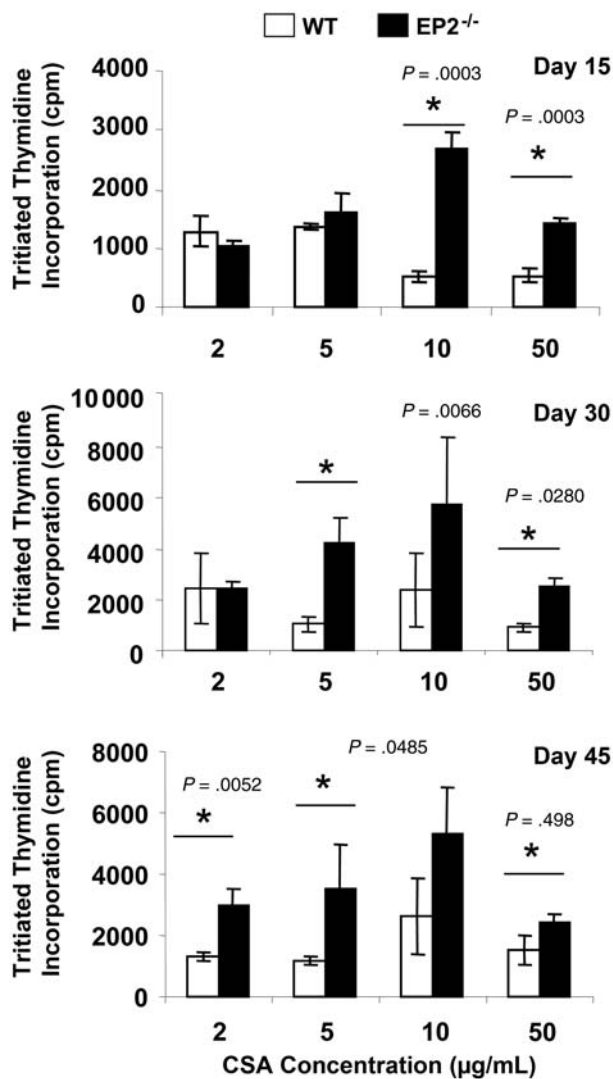


Figure 3. Splenocytes from EP2^{-/-} mice exhibit increased antigen-specific proliferation in response to mycobacterial complete soluble antigen (CSA). Splenocytes were isolated from uninfected and *Mycobacterium tuberculosis* H37Rv-infected EP2^{-/-} and C57BL/6 (wild-type) mice and stimulated with different concentrations of CSA. Proliferation was analyzed after 72 hours using tritiated thymidine. Data shown here is representative of 3 independent experiments with 3 mice in each group and represents the mean ± standard deviation values.

animals do not exhibit impaired lymphoproliferative activities. We next determined if EP2^{-/-} animals induce biased T-cell responses following *M. tuberculosis* infection. To delineate the nature of antigen-specific responses generated in EP2^{-/-} mice, we analyzed the cytokines in splenocyte culture supernatants. Surprisingly, there were no significant differences in the production of IFN- γ , whereas IL-4 was significantly reduced in the cultures from EP2^{-/-} mice (Figure 4A). Interestingly, we noted that IL-17 ($P = .0062$) and interleukin 10 (IL-10) ($P = .0112$) were significantly increased in the cultures from

EP2^{-/-} mice (Figure 4A). These data suggested the possibility that infected EP2^{-/-} animals might have increased levels of T helper 17 (Th17) cells and Tregs.

EP2^{-/-} Animals Induce Increased Th17 and Treg Cell Responses to *M. tuberculosis*

From the preceding data, it was clear that IFN- γ levels in splenocyte cultures from *M. tuberculosis*-infected EP2^{-/-} and WT animals were similar, whereas IL-17 and IL-10 levels were dramatically increased in cultures from EP2^{-/-} animals. Because IL-10 is predominantly produced by Th2 cells and Tregs, and because we detected little IL-4 in the cultures, we considered it most likely that Tregs were the relevant source of IL-10. Thus, to obtain insight into the cellular source of IL-10, we examined the mRNA expression levels of *Gata-3* and *Foxp3*, which are the lineage-specific transcription factors of Th2 cells and Tregs, respectively. Consistent with the cytokine data, expression of *Gata-3* was reduced in EP2^{-/-} mice, whereas expression of *Foxp3* was increased, as compared with WT mice (Figure 4B). This observation suggested that there were increased numbers of Tregs in EP2^{-/-} animals. Tregs also produce Tgf- β , which is often considered to be the most relevant cytokine produced by these cells. Therefore, we next determined the expression of *Tgf- β* . As expected, we observed significantly increased expression levels of *Tgf- β* in EP2^{-/-} animals (Figure 4B). We also analyzed Tgf- β in the culture supernatants of splenocytes rechallenge ex vivo with *M. tuberculosis* complete soluble antigen. The Tgf- β levels were higher in cultures from EP2^{-/-} animals than from WT animals (Figure 4C). To further confirm these data, we next examined Tregs in EP2^{-/-} mice by fluorescence-activated cell sorting (FACS). Splenocytes were isolated from WT and EP2^{-/-} mice at 30 days after infection and stained with fluorescently labeled antibodies for CD4 and CD25 surface markers and the intracellular transcription factor Foxp3. We also evaluated cells for intracellular expression of IL-4, the prototypical Th2 cytokine. EP2^{-/-} mice exhibited a 2-fold increase in the levels of CD4⁺CD25⁺Foxp3⁺ Tregs, as compared with WT mice, whereas these animals had lower levels of IL-4-producing cells (Figure 5). Collectively, these data suggested that infected EP2^{-/-} mice produce increased levels of Tregs. We also evaluated IL-17-producing T helper cells in EP2^{-/-} and WT mice by intracellular staining. T helper cells from EP2^{-/-} mice showed increased levels of IL-17, as compared with WT mice (Figure 5).

Histological Analysis of Granulomatous Lesions

To investigate lung pathology of the infected animals, we examined the histological sections of uninfected and infected EP2^{-/-} and WT mice. Lung sections were stained with hematoxylin and eosin and analyzed by light microscopy. In agreement with the CFU data, we observed higher numbers of granuloma-like structures in EP2^{-/-} mice (Figure 6A) than in

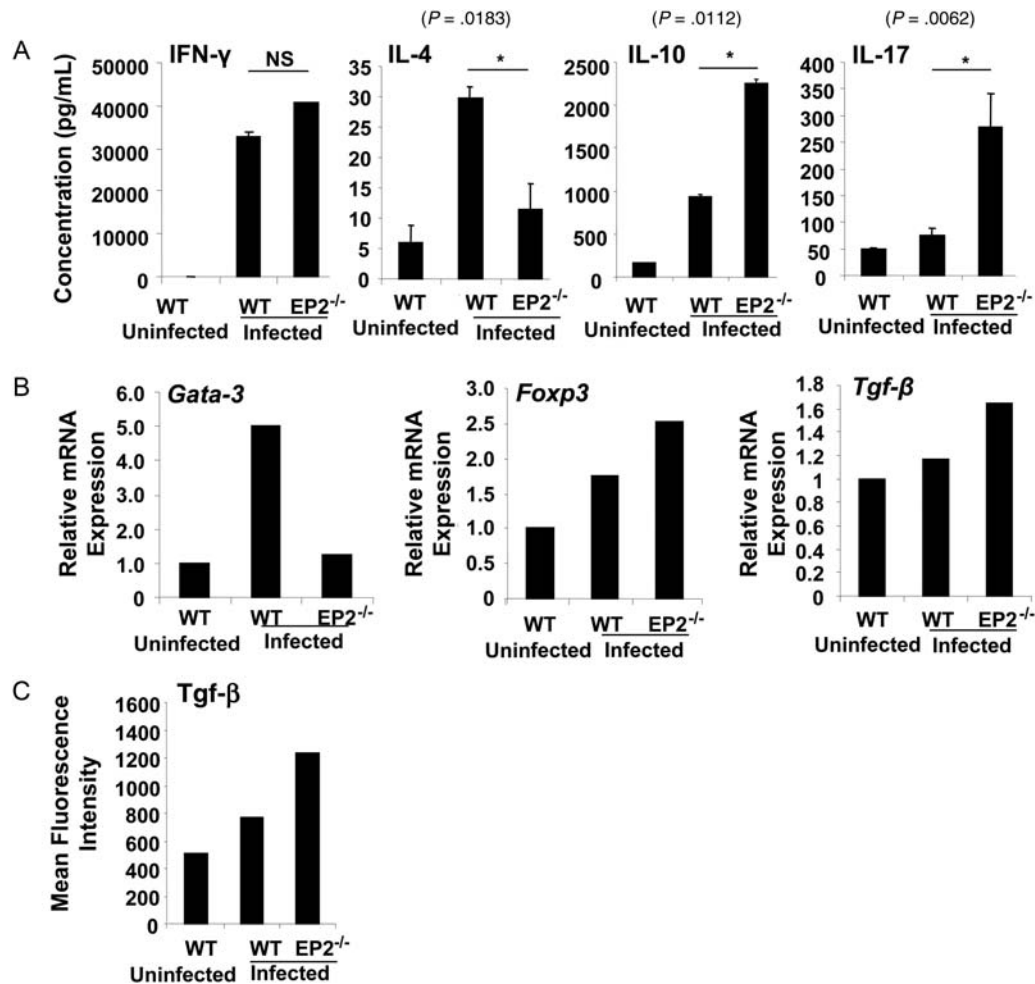


Figure 4. EP2-deficient mice induce regulatory T cells upon infection with *Mycobacterium tuberculosis*. *A*, T helper 1 (IFN- γ), T helper 2 (IL-4 and IL-10), and T helper 17 (Th17) cytokine responses were assayed in the culture supernatants of splenocytes derived from uninfected and infected C57BL/6 (wild-type [WT]) and EP2^{-/-} mice at 48 hours after activation with *M. tuberculosis* H37Rv protein lysate (complete soluble antigen [CSA], 20 μ g/mL). Data shown here are representative of 3 independent experiments, with 3 mice per experiment. Abbreviations: IFN- γ , interferon γ ; IL-4, interleukin 4; IL-10, interleukin 10; IL-17, interleukin 17; NS, not significant. *B*, EP2^{-/-} mice show increased T regulatory cell-specific transcription factor (*Foxp3*) and cytokine (*Tgf- β*) expression. CD4⁺ T cells derived from uninfected and infected C57BL/6 (WT) and EP2^{-/-} mice were analyzed for expression levels of *Gata-3*, *Foxp3*, and *Tgf- β* messenger RNA by real-time polymerase chain reaction. Data are representative of 2 experiments, with 3 mice per group. *C*, Infected EP2^{-/-} mice show increased Tgf- β secretion in the culture supernatants of splenocytes rechallenged ex vivo with *M. tuberculosis* CSA. Data are representative of 2 experiments, with 3 mice per group.

WT mice. We also determined Tgf- β expression in the lung by immunohistochemistry. Consistent with the cytokine data for splenocytes, we found that EP2^{-/-} animals produced dramatically increased levels of Tgf- β in the lung (Figure 6B).

DISCUSSION

It is well known that PGE₂ is an immunomodulatory molecule produced by infected macrophages [3]. PGE₂ has four receptors with several different effects on antigen-presenting, cell-mediated effector T-cell functions and homeostasis [3]. However, the role played by individual PGE₂ receptors on the

modulation of T-cell responses has not been well documented. Our data show that EP2 receptor mRNA expression is selectively modulated in CD4⁺ T cells during *M. tuberculosis* infection. EP2 receptor mRNA expression was highly upregulated at 30 days after infection and correlated with the bacterial burden in lungs and spleen. However, the expression profile of the other 3 EP receptors did not show such a pattern, with EP1 receptor levels being almost undetectable. Because PGE₂ is a ubiquitous mediator of inflammation that affects the systemic response in a receptor-dependent manner, this pattern of expression of the EP2 receptor during *M. tuberculosis* infection of mice appeared relevant to the activity of PGE₂. Prior

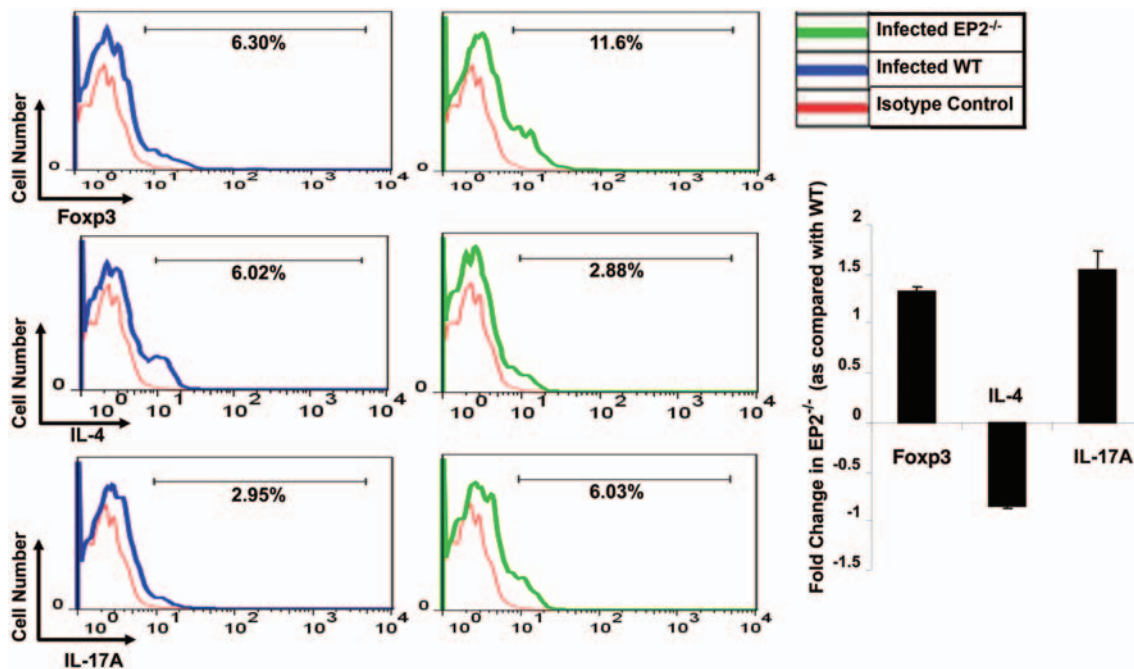


Figure 5. Infected EP2^{-/-} mice produce increased levels of CD4⁺CD25⁺Fcγ3⁺ T regulatory cells (Tregs), increased levels of interleukin 17A (IL-17A)-producing cells, and reduced levels of interleukin 4 (IL-4)-producing cells. Splenocytes were isolated from uninfected and infected C57BL/6 (wild-type [WT]) and EP2^{-/-} mice (30 days after infection) and were stained with fluorescently labeled antibodies for CD4 and CD25 (for Tregs) surface markers, and intracellular staining was performed for the Fcγ3 transcription factor and IL-17A and IL-4 cytokines. Data shown here are representative of 3 independent experiments, with 3–5 mice per experiment. Data are expressed as fold change in expression levels (\pm standard deviation).

studies have shown that EP2 modulates adaptive immune responses by influencing DC function and antigen-specific T-cell generation and by promoting Th2-cell responses [3]. Because Th2 cells inhibit T helper 1 (Th1) cells, the effect of EP2 would be expected to suppress host-protective immune responses. In this context, it has been well documented that virulent *M. tuberculosis* strains induce exuberant Th2-type immune responses [17–19]. Furthermore, animals unable to mount Th2 responses, such as IL-4^{-/-} or Stat-6^{-/-} mice, are partially resistant to the development of tuberculosis [20]. We found that EP2 receptor-deficient mice exhibit increased susceptibility to *M. tuberculosis* infection, as shown by an increased bacterial load in the lungs and spleen of these animals as compared with WT mice.

Our recent findings have shown that EP2-mediated signaling specifically inhibits activation-induced cell death in Th2 cells [7]. Consistent with these studies, we were unable to detect differences in IFN- γ production, whereas IL-4 production by *M. tuberculosis* antigen-specific CD4⁺ T cells was decreased in EP2^{-/-} mice. Such a Th1 vs Th2 cytokine profile would be expected to render EP2^{-/-} animals less susceptible to bacterial infection than WT mice. Indeed, previous studies have shown enhanced clearance of several gram-negative [21] and gram-positive [22] bacteria from the lungs of EP2^{-/-}

mice. It was, therefore, surprising to observe that these animals had increased susceptibility to *M. tuberculosis* infection. A second surprising result of our studies was that, despite such a reduction in Th2 cytokine responses, splenocytes from EP2^{-/-} mice exhibited enhanced proliferative responses against *M. tuberculosis* antigens. This finding is consistent with previous reports showing that EP2 receptors primarily inhibit mixed lymphocyte reactions through direct effects on T cells [23]. Our analysis of cytokines and Th-cell subsets further indicated that infected EP2^{-/-} mice developed enhanced Th17 and Treg responses. In this context, it is important to note that Tregs inhibit the activation and proliferation of antigen-specific T cells in a Tgf- β -dependent manner. However, Tgf- β is unable to inhibit Th17-cells and instead augments Th17-cell differentiation [24, 25]. Thus, the immune response against *M. tuberculosis* appears to be regulated by PGE₂-EP2 axis in a manner that is distinct from the response generated against most other bacterial organisms. This might be attributed to the chronicity of the *M. tuberculosis* infection, in which Tregs play an important role.

The roles of Th17 cells and IL-17 in the pathogenesis of tuberculosis have been extensively debated. Although studies have shown a protective role of IL-17 during *M. tuberculosis* infection, Th17 cells and Th17-derived cytokines also mediate

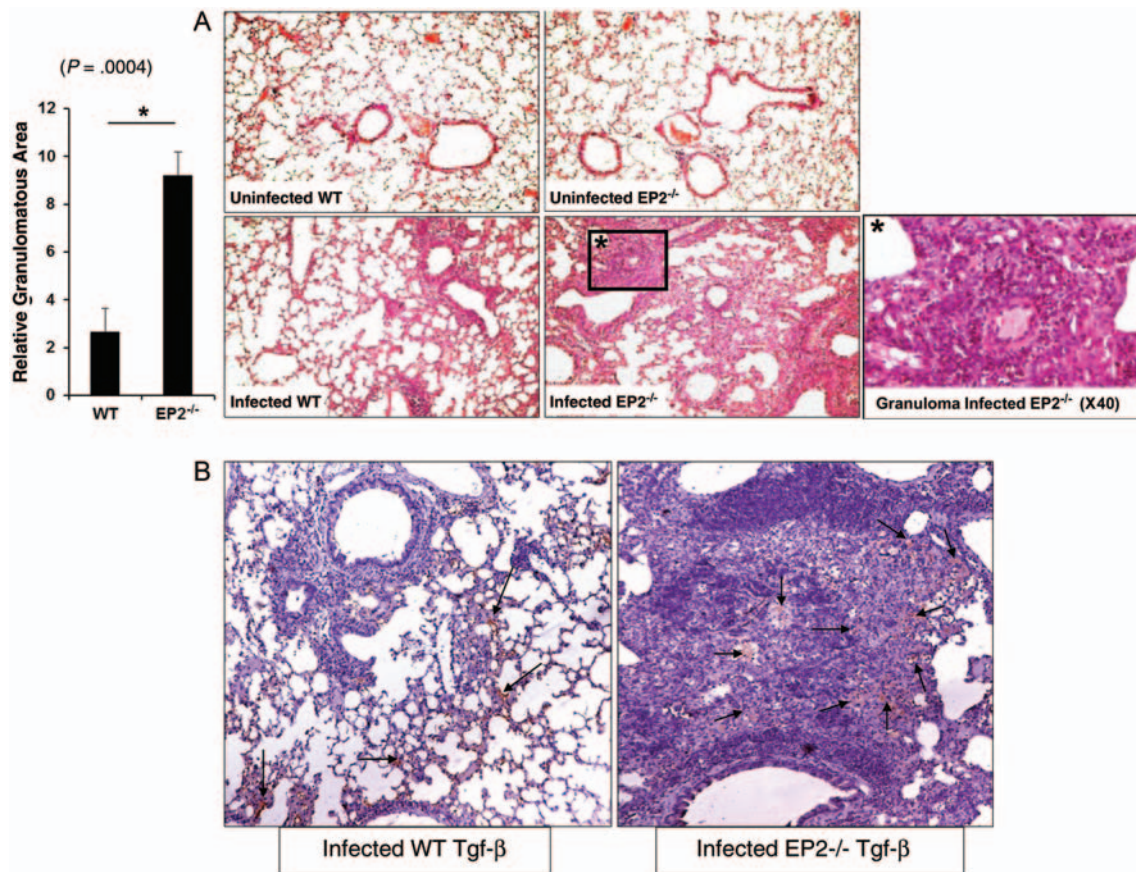


Figure 6. Differences in granulomatous responses to *Mycobacterium tuberculosis* infection in EP2^{-/-} and C57BL/6 (wild-type [WT]) mice. *A*, Granuloma formation in EP2^{-/-} and WT mice after low-dose *M. tuberculosis* aerosol infection was analyzed 30 days after infection. Sections of formalin-fixed and paraffin-embedded lung tissue of uninfected and infected WT and EP2^{-/-} mice were used to visualize hematoxylin and eosin staining. Data are presented as relative granulomatous area image compared per fixed field of view for the entire lung and represent the mean ± standard error of the mean values. One representative image of 3 independent experiments is shown. Original magnification, ×10, unless otherwise noted. *B*, Immunohistochemical staining of cytokine Tgf-β in infected WT and EP2^{-/-} mice 30 days after infection. Photographs are representative images from lung tissue sections from each group of mice. The staining for Tgf-β in these photographs is depicted in red color and marked by arrows. Original magnification, ×10.

inflammation and tissue damage in autoimmune diseases and infections [26]. During *M. tuberculosis* infection of genetically susceptible animals, IL-17 promotes granuloma formation as well as neutrophil accumulation and tissue damage [26]. Neutrophils provide a favorable environment for bacterial replication because they are the predominant cell type infected in tuberculosis patients [27]. Tgf-β has been shown to be involved in the development and/or consequences of granuloma formation during *M. tuberculosis* infection [28]. Hence, the increased secretion of IL-17 and Tgf-β by CD4⁺ T cells in EP2^{-/-} mice as compared with WT mice might contribute to the increased lung pathology in these animals. However, additional studies are required to delineate the precise mechanism by which EP2 regulates IL-17 and Tgf-β production during *M. tuberculosis* infection.

Based on published studies [4, 5, 7–9, 16, 22, 29] and the findings reported here, we propose the following role of PGE₂

and EP2 during infection. Upon exposure to antigen or infection by typical bacteria, macrophages produce PGE₂, which engages with EP2 and EP4 on DCs and EP2 on T cells to keep the immune response in balance. Thus, for most bacteria, EP2 deficiency results in increased resistance to infection. In the case of tuberculosis, the microorganisms upregulate EP2 expression on CD4⁺ T cells, and engagement of this receptor with PGE₂ enhances IL-4–producing cells, which facilitates chronic infection. Consequently, infection of EP2^{-/-} animals with *M. tuberculosis* results in reduced levels of antigen-specific Th2 cells in the face of unaltered levels of Th1 cells and increased levels of Tregs and Th17 cells. As a result of these alterations in Th-cell responses, disease progression in EP2-deficient animals is enhanced (Figure 7). We speculate that the effects of EP2 expression on other microorganisms that cause a chronic infection might be similar to those described here for *M. tuberculosis* infection.

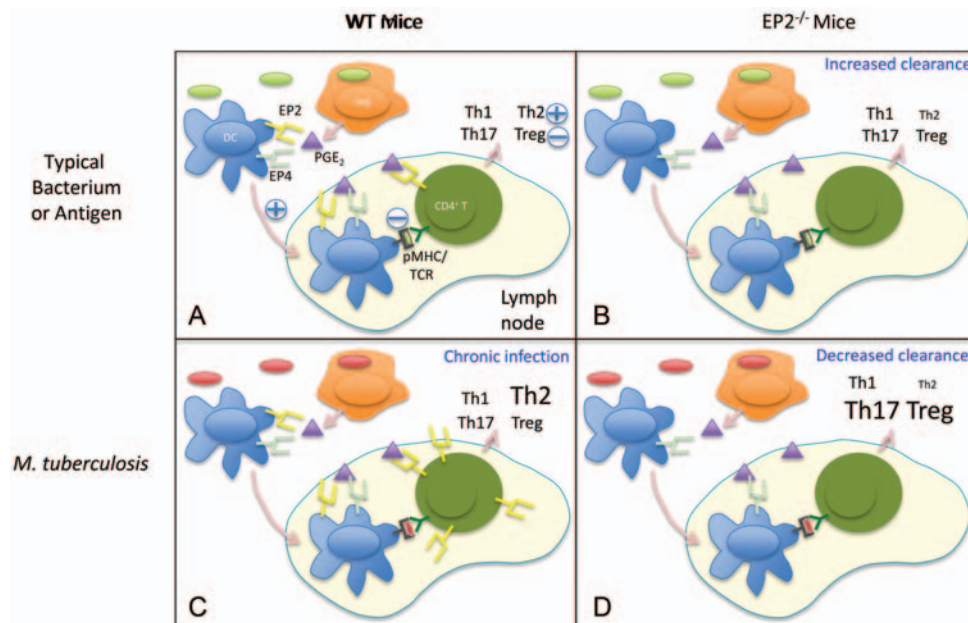


Figure 7. Proposed model for the role of EP2 in PGE₂-mediated activities during T-cell responses against bacterial infection. *A*, In wild-type (WT) mice exposed to typical bacteria or antigens, PGE₂ secreted by macrophages and dendritic cells (DCs) acts mainly on EP2 and EP4 receptors to modulate DC migration and antigen presentation to naive CD4⁺ T cells in the peripheral lymph nodes and on EP2 receptors expressed by CD4⁺ T cells to modulate the generation of T-cell responses. Effects of PGE₂ are indicated by + and – symbols. Font sizes of distinct T helper (Th) subsets represent an estimation of their prevalence. pMHC/TCR, peptide-MHC/T cell receptor. *B*, Following infection with typical bacteria, EP2-deficient mice show increased bacterial clearance and reduced Th2 type responses. *C*, In chronic bacterial infections such as tuberculosis, induction of EP2 on CD4⁺ T cells results in increased Th2 responses and bacterial persistence. *D*, Despite a reduction in Th2 responses, EP2-deficient mice show reduced bacterial clearance of *Mycobacterium tuberculosis* organisms. This is likely due to an increase in Th17 and T regulatory cell (Treg) responses, which leads to increased bacterial persistence in granulomas and enhanced bacterial burden.

In summary, our data have shown that absence of the PGE₂ receptor EP2 increases susceptibility to *M. tuberculosis* infection. Although reduced Th2 responses are generated in the absence of the EP2 receptor, increased levels of immunosuppressive cytokines, such as IL-10, produced by Tregs lead to a reduction in protective immune responses in these animals. This effect is reinforced by increased levels of the proinflammatory cytokine IL-17 produced by antigen-specific CD4⁺ T cells in EP2-deficient mice. Thus, the EP2 receptor mediates host resistance during *M. tuberculosis* infection and might serve as a novel target for immunotherapy.

Notes

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