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Interdependence between interleukin-1 and tumor necrosis factor controls TNF-dependent effector functions during Mycobacterium tuberculosis infection

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Summary
The interleukin-1 receptor I (IL-1RI) is critical for host resistance to Mycobacterium tuberculosis (Mtbd), yet the mechanisms of IL-1RI-mediated pathogen control remain unclear. Here, we show that without IL-1RI, Mtbd-infected newly recruited Ly6CHigh myeloid cells failed to up-regulate

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Supplemental data include six figures and supplemental materials and methods and can be found with this article online at http://

Author Contributions
N.C.D.P. and D.M.S. conceived the study, N.C.D.P. and S.S. conducted most of the experiments, T.D. performed experiments and analyzed plasmid-based Mtbd replication in vivo, T.P. and D.W.R. provided advice on and shared reagents for bone marrow transplantation studies and foamy virus production, Y.I., D.S., and K.U. shared critical reagents, K.U., N.C.D.P., and D.M.S. guided the study and analyzed the data, N.C.D.P. and D.M.S. wrote the manuscript. All authors discussed the results and commented on the manuscript.
tumor necrosis factor receptor I (TNF-RI) and to produce reactive oxygen species, resulting in compromised pathogen control. Furthermore, simultaneous ablation of IL-1RI and TNF-RI signaling on either stroma or hematopoietic cells led to early lethality, indicating non-redundant and synergistic roles of IL-1 and TNF in mediating macrophage-stroma cross-talk that was critical for optimal control of Mtb infection. Finally, we show that even in the presence of functional Mtb-specific adaptive immunity, the lack of IL-1α and not IL-1β led to an exuberant intracellular pathogen replication and progressive non-resolving inflammation. Our study reveals functional interdependence between IL-1 and TNF in enabling Mtb control mechanisms that are critical for host survival.

Introduction

*Mycobacterium tuberculosis* (*Mtb*) is one of the leading causes of human mortality associated with a single infection agent world-wide. The stereotypic tissue response to infection with *Mtb* is the formation of granulomas, a focal inflammatory response where cell-cell cross-talk coordinates cell movement, retention, and function of the granuloma structure (Ramakrishnan, 2012). Previous studies have identified a number of factors and cell subsets of the innate and adaptive immune systems that are critical for host resistance to *Mtb* (Cooper, 2009; O’Garra et al., 2013; Ramakrishnan, 2012). It has been demonstrated that mice individually deficient in tumor necrosis factor (TNF) or tumor necrosis factor-receptor I (TNF-RI) (Flynn et al., 1995) are extremely susceptible to low-dose aerosol *Mtb* infection due to the fundamental role of TNF-RI signaling in maintaining granuloma structure and enabling cell-intrinsic mechanisms of *Mtb* control (Cantini et al., 2015; Clay et al., 2008; Diedrich et al., 2013). It has also been demonstrated that mice deficient in interleukin-1α and IL-1β or IL-1RI (Fremond et al., 2007; Yamada et al., 2000) are extremely susceptible to low-dose aerosol *Mtb* infection. However, it is unclear why TNF, which is abundantly expressed in the lungs of IL-1-deficient mice, fail to control *Mtb* in the absence of IL-1 signaling.

IL-1α and IL-1β are non-homologous protein members of the IL-1 family cytokines with pleiotropic roles in host immunity, inflammation, and homeostasis (Dinarello, 2009; Garlanda et al., 2013). Both IL-1α and IL-1β trigger identical biological responses after binding to the IL-1RI (Dinarello, 2011). In the context of *Mtb* infection, it remains controversial whether IL-1α or IL-1β plays redundant or non-redundant roles in mounting optimal pathogen control. For instance, one study demonstrates that in vivo neutralization of IL-1α, but not IL-1β, renders mice highly susceptible to *Mtb* (Guler et al., 2010). In another study, mice deficient in IL-1β are shown to be as much susceptible to *Mtb* as IL-1RI-deficient mice and succumb to infection within 4 weeks (Mayer-Barber et al., 2010). Moreover, it has been also proposed that during *Mtb* infection, both IL-1α and IL-1β may cooperate in establishing host resistance to *Mtb* (Mayer-Barber et al., 2011).

To evaluate functional interdependence between the TNF and IL-1 pathways and the individual roles of IL-1α and IL-1β ligands in the control of *Mtb* infection, we performed bone marrow cross-transplantations between wild-type mice and mice deficient in proteins of the IL-1, TNF, or both signaling pathway and analyzed resistance and immuno-pathology.
of these chimeric mice using a low-dose aerosol infection with *Mtb*. We found that IL-1 and TNF play non-redundant and synergistic roles in mediating cross-talk between hematopoietic and stoma cells that is critical for host resistance to *Mtb*. We further demonstrate that in the presence of functional *Mtb*-specific adaptive immunity, the lack of IL-1α and not IL-1β led to an exuberant intracellular pathogen replication, progressive non-resolving inflammation, and earlier lethality. Together, these data provide mechanistic insights into compartment-specific IL-1RI- and TNF-RI signaling during pulmonary *Mtb* infection, their functional interdependence for enabling mechanisms of *Mtb* control, and the critical role of IL-1α for long term host survival during *Mtb* infection.

**Results**

**IL-1RI on hematopoietic cell is required for host resistance to *Mtb***

Although WT mice or WT mice transplanted with WT bone marrow did not show susceptibility to *Mtb* infection, *Il1r1*−/− mice, WT mice transplanted with *Il1r1*−/− bone marrow cells, and *Il1r1*−/− mice transplanted with *Il1r1*−/− bone marrow, succumbed to *Mtb* infection, indicating that IL-1RI on hematopoietic cells is critical for host resistance to *Mtb* (Figure 1A). In agreement with previous reports (Fremond et al., 2007; Mayer-Barber et al., 2011), the analysis of expression of inflammatory cytokines and chemokines 30 days p.i. showed that TNF, IL-6, and key pro-inflammatory chemokines were expressed at much higher amounts in the lungs of *Mtb*-infected *Il1r1*−/− or *Il1ab*−/− mice, when compared to WT, *Il1a*−/−, or *Il1b*−/− mice (Figure 1B), demonstrating that without functional IL-1RI signaling, *Mtb* infection leads to exuberant inflammation and TNF expression fails to confer protection. In order to understand why IL-1RI-deficient mice succumb to *Mtb* in the presence of TNF, we next infected WT mice transplanted with bone marrow cells from either WT or *Il1r1*−/− mice with an *Mtb* strain that expresses the red fluorescent protein mCherry and analyzed expression of TNF-RI and reactive oxygen species (ROS) production in *Mtb*-infected cells 17 days p.i. This analysis showed that *Il1r1*−/− mice contained higher numbers of *Mtb*-infected cells in the lungs (Figures 1C and S1A). This gain in *Mtb*-infected cells in *Il1r1*−/− mice was due to the higher numbers of cells that showed autofluorescence in the 488 channel (488-Auto+, Figures 1D–F and Figure S1B), and over 95% of these cells express CD64 and CD11b monocytic markers and could be further divided onto distinct CD11c+ (myeloid dendritic cells and alveolar macrophages) and CD11c− (newly recruited monocytes, macrophages, and PMNs) populations (Figures 1D, S1C; (Wolf et al., 2007)). Using ImageStream technology, we next confirmed that all cells that appeared mCherry-positive by flow cytometry, including those autofluorescent in 488 channel, were genuinely infected with mCherry-expressing *Mtb* bacilli (Figure 1E). Because the gain in *Mtb*-infected cells in *Il1r1*−/− mice, compared to WT mice, was primarily attributable to 488-Auto+ CD64+CD11c− myeloid cells (Figures 1F and S1B), we next analyzed the cellular composition of this population using antibodies specific to Ly-6G and CD11b. This analysis showed that this population was composed of Ly6Ghigh (and CD11bhigh; PMNs and Ly6Glow (and CD11blow; newly recruited monocytes and macrophages) cells (Figure S1C). CD64+CD11c−Ly6Ghigh cells represented 33% and 27% of all *Mtb*-infected cells in WT and *Il1r1*−/− mice respectively (Figure 1G). In contrast, *Mtb*-infected CD64+CD11c−Ly6Glow cells were nearly absent in WT mice and their number was significantly higher in *Il1r1*−/−
mice (4% and 12.5%, respectively). The analysis of cell-specific properties of these two populations showed that upon *Mtb* infection *in vivo*, the 488-Auto− phenotype associated with uniformly low TNF-RI expression, ROS production, and pathogen burden in both WT and *Il1r1−/−* mice (Figures 1H–M, 488-Auto− populations). Upon acquisition of 488-Auto+ phenotype, in WT mice CD64+CD11c−Ly6Ghigh cells activated high amounts of surface TNF-RI expression, ROS production, and exhibited low *Mtb* burden indicating effective pathogen control (Figures 1H–J). In contrast, this population in *Il1r1−/−* mice activated significantly lower amounts of TNF-RI, ROS, and failed to control the burden of *Mtb* at a single cell level (Figures 1H–J). The response of CD64+CD11c−Ly6Glow cells to *Mtb* infection was qualitatively different and, although activating high amounts of TNF-RI and ROS upon transition to autofluorescent phenotype, this cell population failed to control *Mtb* burden in both WT and *Il1r1−/−* mice, demonstrating that this cell type, consistent with newly recruited inflammatory monocytes, is intrinsically permissive to *Mtb*, and failed to establish bactericidal state even in WT mice. Importantly, this cell population was only marginally present in WT mice, while its proportion was significantly higher in *Il1r1−/−* mice (Figure 1G). Analysis of freshly isolated lung mononuclear cells showed that higher numbers stained positively with necrotic cell dye in *Il1r1−/−* mice, compared to WT mice (Figures S1E–F). Furthermore, administration of propidium iodide into *Mtb*-infected mice revealed extensive distribution of necrotic cells in granulomas of *Il1r1−/−* mice but not in WT mice at 30 days p.i. (Figure S1H). At this time point, the *Mtb* burden was 3 order of magnitude higher in *Il1r1−/−* mice, compared to WT mice (Figure S1G), and acid-fast staining of lung sections revealed high number of *Mtb* bacilli localized in cells with interstitial monocyte morphology (Figure 1N).

Together, these data indicate that the lack of IL-1RI on hematopoietic cells results in inability of CD64+CD11b+CD11c−Ly6Ghigh *Mtb*-infected cells to upregulate protective amounts of TNF-RI and trigger ROS production, resulting in compromised cell-intrinsic *Mtb* control. This further associates with the recruitment of *Mtb*-permissive CD64+CD11b+CD11c−Ly6Glow interstitial monocyte cell pool, exuberant *Mtb* replication, and early lethality despite abundant TNF expression.

**Monocyte-stroma cross-talk through IL-1RI and TNF-RI is required for optimal resistance to *Mtb***

In order to define the source of IL-1 and TNF and the directionality of host-protective IL-1RI and TNF-RI signaling, we cross-transplanted WT mice with bone marrow from TNF or TNF-RI-deficient animals. In agreement with earlier studies, mice deficient in TNF or TNF-RI in hematopoietic cells were highly susceptible to *Mtb* (Figure 2A) (Flynn et al., 1995). We further found that *Tnf−/−* and *Tnfr1−/−* mice transplanted with WT bone marrow were also more susceptible to *Mtb* infection, compared to WT mice transplanted with WT bone marrow, demonstrating that TNF from and TNF-RI on stromal cells were also needed for optimal protection from *Mtb* (Figure 2A). Treatment of bone marrow-differentiated macrophages or immortalized mouse lung epithelial cells (MLg) with IL-1β, TNF, and/or heat-killed *Mtb* (as likely agonists during progressive *Mtb* infection), showed that combination of IL-1β+TNF or heat-killed *Mtb*+TNF resulted in the most potent activation of
TNF and IL-1α, among other cytokines and chemokines in both cell types (Figures 2B and 2C).

Because IL-1α can function as a membrane-bound cytokine, we treated bone marrow macrophages with LPS or heat-killed Mtb and stained cells with anti-IL-1α Abs. This staining showed that both treatments produced IL-1α-specific staining on non-permeabilized cells (Figure 2D). To analyze if membrane-IL-1α is biologically active, we exposed reporter cell line LA4 (that produces CXCL1 chemokine in response to recombinant IL-1 (Figure 2E)) to bone marrow macrophages from WT, Casp1−/−, or Casp1−/−Il1a−/− (as negative control) mice treated to produce membrane-bound IL-1α, and confirmed that membrane-bound IL-1α on macrophage cells was biologically active and its CXCL1-stimulating activity on LA4 cells could be blocked by anti-IL-1α antibodies (Figure 2F). Moreover, and similar to cell treatment with IL-1β+TNF and heat-killed Mtb+TNF (Figure 2C), MLg cells treated with a combination of TNF and macrophage cells presenting membrane-bound IL-1α led to the most potent activation of cytokines and chemokines when compared to individual treatments (Figure 2G). To ultimately test whether simultaneous IL-1RI and TNF-RI signaling to and from stromal cells is required for host resistance to Mtb in vivo, we cross-transplanted Il1r1−/− or Il1r1−/−Tnfr1−/− mice with WT bone marrow. While Il1r1−/− mice transplanted with WT bone marrow survived up to 100 days p.i (Figure 2H), WT bone marrow failed to rescue Il1r1−/−Tnfr1−/− mice, which succumbed to infection with median survival of 38 days. This median survival was significantly lower than that observed in Tnfr1−/− mice transplanted with WT bone marrow (58 days), demonstrating that both IL-1RI and TNF-RI signaling on stroma cells are synergistically required for resistance to Mtb.

**IL-1α mediates host resistance to Mtb in a non-redundant fashion**

To define the role of individual IL-1RI ligands during the course of Mtb infection, we infected mice deficient in IL-1RI, IL-1α (Horai et al., 1998), IL-1αβ (Horai et al., 1998), or IL-1β (Shornick et al., 1996) with Mtb. Il1r1−/− and Il1ab−/− mice succumbed to infection with a median survival of 28 and 35 days (Figure 3A). There was a significant divergence in the survival phenotype between Il1a−/− and Il1b−/− mice, which succumbed to Mtb infection with a median survival of 95 and 152 days p.i., respectively (Figure 3A). These results were reproduced with more virulent Mtb Erdman strain (Figures S2A and S2B). Bacterial burden analysis in the lungs of WT mice showed a plateau of ~10^6 CFU per lung at 30 days p.i., and infection was controlled at this level for over 200 days. In contrast, Il1r1−/− and Il1ab−/− mice reached a bacterial burden of 10^8 CFU by 30 day p.i., and Il1a−/− mice reached 10^8 CFU by 90 days p.i. (Figure 3B). The Mtb burden in the lungs of Il1b−/− and Casp1−/−Il1a−/− mice was not different from mice in the control group (Figure S2C). Hematoxylin and eosin and acid-fast staining of lung sections revealed that by 60 days p.i. in WT mice, defined granulomas containing few Mtb bacilli were formed. In contrast, in Il1a−/− mice lungs contained large inflammatory lesions with highly diffuse structure and cells with numerous bacilli were dispersed throughout the lung parenchyma (Figure 3C). Evaluation of lung histopathology revealed worsening of histopathology score (Figures 3D and S2D) and progressive decline in inflammation-free airway space in the lungs of Il1a−/− mice (Figure 3E). Collectively, our analyses show that although within the first 5 weeks p.i. IL-1RI ligands can play compensatory roles in...
mediating host resistance to *Mtb* and mice deficient in either IL-1α or IL-1β survive beyond 35 days p.i., IL-1α and IL-1β play non-redundant roles at later times p.i. when deficiency in IL-1α compromises host resistance to a significantly greater degree, compared to deficiency in IL-1β.

**Mice deficient in IL-1 ligands develop functional *Mtb*-specific adaptive immunity**

The delayed susceptibility of *Il1a*−/− mice to pulmonary *Mtb* infection could be explained by a requirement of IL-1α for an effective adaptive immune response (Ben-Sasson et al., 2009). To test this hypothesis, we used MHC class I and II tetramers to track CD8+ and CD4+ T cells recognizing *Mtb* immunodominant MHC class I (TB10.44-11:Kb) and MHC class II (ESAT-64-17:Ab) -restricted T cell responses in the lungs of WT, *Il1a*−/−, and *Il1ab*−/− mice. At 30 days and 60 days p.i., this analysis revealed similar frequencies of tetramer-binding CD4+ and CD8+ T cells in all groups (Figures 4A–D, S3). To determine the frequency of IFN-γ-producing T cells *in vivo*, we also performed direct *ex vivo* intracellular IFN-γ staining on freshly isolated lung lymphocytes in the absence of *in vitro* stimulation (Shafiani et al., 2010). This analysis revealed significantly higher proportions of IFN-γ-producing cells within the tetramer-binding CD4+ and CD8+ populations of *Il1a*−/− and *Il1ab*−/− mice, compared to control group, at 30 days p.i. (Figure 4B, D). The higher frequency of IFN-γ-producing T cells in *Il1a*−/− and *Il1ab*−/− mice demonstrate that T cells from these strains are not intrinsically deficient in their ability to express IFN-γ *in vivo*. *In vitro* stimulation of lung lymphocytes, freshly isolated from *Mtb*-infected WT and *Il1a*−/− mice, with the anti-CD3 and anti-CD28 Abs, ESAT-64-17, or TB10.44-11 peptides did not reveal any differences in the proportions of IFN-γ or TNF-producing CD4+ and CD8+ T cells between the groups (Figure 4E, F). Therefore, these analyses show that the development of *Mtb*-specific adaptive immunity does not depend on IL-1 cytokines.

**The lack of IL-1α-IL-1RI signaling leads to a non-protective highly inflammatory state and premature lethality**

Gross examination of the *Mtb* infected mice showed that by 90 days p.i., the wet lung weight for *Il1a*−/− mice exceeded that for WT animals by 180% (P<0.01, Figure 5A), and the number of mononuclear cells harvested from the lungs of *Il1a*−/− mice was significantly greater than from the WT mice both at 60 and 90 days p.i. (P<0.01, Figure 5B). The frequency of Ly-6G+Ly-6C+ polymorphonuclear leukocyte (PMNs) population at this late time point was lower in *Il1a*−/− mice (1.88%) compared to WT animals (3.14%, P<0.05, Figure 5C), excluding the possibility that the pulmonary pathology in *Il1a*−/− mice is associated with neutrophilic inflammation. This analysis also revealed that the majority of leukocytes purified from the lungs of *Il1a*−/− mice expressed Ly-6C marker, associated with inflammatory monocytes, macrophages, and dendritic cells (DCs) (De Trez et al., 2009; Mayer-Barber et al., 2011; Swirski et al., 2009). To better define the *in vivo* activation status of monocytes accumulated in the lungs of WT and *Il1a*−/− mice after *Mtb* infection, we assessed isolated lung cells for expression of CD11b and CD11c, as well as Ly-6C and CD40 as markers associated with cellular activation. We observed a dramatic increase in Ly-6C expression by both CD11b+ and CD11c+ cell populations isolated from *Il1a*−/− mice, compared to WT animals (P<0.01, Figure 5D). In addition, the population of CD11c+Ly-6C+ monocytes observed in WT was absent in *Il1a*−/− mice (population a,

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Figure 5D). The analysis of CD40 expression on lung leukocytes showed that after Mtb infection, the proportion of highly activated CD11c<sup>high</sup>CD40<sup>high</sup> monocytes was over 2-fold higher in Il1a<sup>−/−</sup> mice compared to WT animals (Figure 5E). Furthermore, a distinct population of CD11c<sup>high</sup>CD40<sup>low</sup> monocytes that was observed in WT animals was completely missing from the lungs of Il1a<sup>−/−</sup> mice (Population b, Figure 5E).

Next, we generated bone marrow chimeras in which lethally irradiated WT mice were transplanted with either WT or Il1a<sup>−/−</sup> bone marrow cells. Five months after hematopoietic reconstitution, chimeric mice were infected with Mtb and Ly-6C and CD40 marker expression on their CD11c<sup>+</sup> cells were analyzed 90 days p.i.. We observed that CD11c<sup>+</sup> cells in chimeric mice recapitulated the phenotypes observed in mice from which the donor bone marrow cells were derived (Figure 5F). Collectively, these data show that in IL-1α-deficient mice, the higher inflammatory state does not correlate with improved resistance to Mtb but rather is associated with progressive non-resolving monocytic inflammation that constrains free airway space and leads to premature lethality.

**Restoration of IL-1α expression in CD11c<sup>+</sup> cells of Il1a<sup>−/−</sup> mice alleviates inflammation and improves survival after Mtb infection**

To determine whether and what kind of Mtb-infected cells express IL-1α in the lungs of Mtb-infected mice, we analyzed sections of lungs from Mtb-infected mice stained with fluorescent IL-1α-specific antibody and polyclonal Mtb-specific antibody or antibodies for various cellular markers by confocal microscopy (Di Paolo et al., 2009). At 60 days p.i., those cells that stained positive for Mtb antigens also stained positive for IL-1α (Figure 6A), and IL-1α-staining co-localized with CD11c<sup>+</sup> and CD11b<sup>+</sup> cells but not with CD3<sup>+</sup> or Gr1<sup>+</sup> cells (Figure 6B).

Because the majority of lung leukocytes including alveolar macrophages, immature DC, and activated DCs, express CD11c, we next determined whether the restoration of IL-1α expression specifically in CD11c<sup>+</sup> cells would suffice to rescue the susceptibility of Il1a<sup>−/−</sup> mice to Mtb infection. We utilized a foamy virus vector-based stable gene transfer approach (Josephson et al., 2002) (Figure S4A), where mouse IL-1α gene was expressed under the control of a minimal Itgax promoter (Ni et al., 2009). CD11c<sup>high</sup>CD40<sup>low</sup> monocyte population was restored in Mtb infected Il1a<sup>−/−</sup> mice transplanted stem cells transduced with Itgax-IL-1α virus (Figure 6C) but not Itgax-stop-IL-1α virus (Figure 6D). Importantly, the median survival of Il1a<sup>−/−</sup> mice transplanted with Itgax-stop-IL-1α vector-transduced stem cells was only 55 days, while 8 out of 9 Il1a<sup>−/−</sup> mice transplanted with hematopoietic stem cells transduced with Itgax-IL-1α virus mice survived beyond 80 days after Mtb infection (P < 0.001, Figure 6E). Therefore, our data show that cell-type-specific restoration of IL-1α expression in CD11c<sup>+</sup> cells in Il1a<sup>−/−</sup> mice results in lung cell phenotypes comparable to WT mice, and prolonged survival after Mtb infection.

**IL-1α is required for intracellular control of Mtb replication at a single cell level in vivo via cell extrinsic mechanism**

To reveal the mechanism of IL-1α-mediated control of Mtb resistance, we analyzed the dynamics of Mtb replication *in vivo*. Quantification of Mtb bacilli on acid-fast stained
sections of lungs revealed that the number of bacilli per high power view field rapidly increased and peaked by 30 days p.i. in all groups (Figure 7A). Of note, although granulomatous areas in the lungs expanded continuously over time (Figure 3D, E), by 60 days p.i., the number of bacilli per view-field drastically declined in WT mice and remained low until 100 days p.i., implying the effective control of *Mtb*. In contrast, the number of bacilli in lung sections was significantly higher for *Il1a*<sup>−/−</sup> and *Il1ab*<sup>−/−</sup> mice at 30 days p.i., compared to WT animals (Figure 7A), suggesting that these mice were deficient in their ability to control *Mtb* replication during early infection. Of note, as in WT animals, the number of bacilli per view-field in *Il1a*<sup>−/−</sup> mice declined to a similar degree between days 30 and 60 p.i., but it increased significantly again by 100 days p.i.

Detailed evaluation of lung sections of *Il1a*<sup>−/−</sup> and WT mice at day 60 p.i. revealed that in *Il1a*<sup>−/−</sup> mice, more than 80% of individual *Mtb*-infected cells contained over 4 bacilli per cell, while in WT animals, only 45% of infected cells contained over 4 bacilli (Figure 7B, C). Furthermore, electron microscopy analysis showed that *Mtb* bacilli in *Il1a*<sup>−/−</sup> mice could be found in clusters (Figures 7D and S5), suggesting bacterial replication *in vivo*. Using a previously described “*Mtb* replication clock” approach to quantify the rate of *Mtb* replication *in vivo* (Gill et al., 2009), we calculated *Mtb* replication rates in *Il1a*<sup>−/−</sup> and WT mice. Between days 1 and 11 post infection, the *Mtb* population in the lungs of *Il1a*<sup>−/−</sup> mice replicated at a rate of 0.77 per day, corresponding to a 31.17 hour generation time. In contrast *Mtb* population in the lungs of WT mice replicated at a significantly slower rate of 0.55 per day, corresponding to 43.64 hours generation time (P<0.01, Figure 7E).

We hypothesized that if the defect in *Mtb* replication control in *Il1a*<sup>−/−</sup> mice is entirely cell-intrinsic, it should be possible to be reproduced *in vitro*. We differentiated macrophages from the bone marrow of WT or *Il1a*<sup>−/−</sup> mice, infected them with *Mtb*, and analyzed the bacterial burden over time. We also treated *Mtb*-infected cells with IL-1α, IFN-γ, or a combination of both, to determine whether these cells were deficient in restricting *Mtb* replication even in the presence of these exogenous cytokines. We found that *Mtb* replicated equally efficiently in both WT and *Il1a*<sup>−/−</sup> macrophages. Furthermore, in agreement with an earlier study, IFN-γ was highly effective at restricting *Mtb* replication *in vitro* (MacMicking et al., 2003), and WT and *Il1a*<sup>−/−</sup> macrophages were equally responsive to IFN-γ and exhibited reduced *Mtb* CFUs (Figures S5B, C). The addition of IL-1α to *Mtb*-infected cells did not restrict bacterial growth. Taken together these data show that *Il1a*<sup>−/−</sup> mice fail to control *Mtb* replication at a single-cell level and this inability to control *Mtb* replication is not cell intrinsic.

**Discussion**

In this study we have analyzed the functional role of the compartment-specific IL-1RI and TNF-RI signaling and the individual contribution of IL1-RI ligands in mediating host resistance to pulmonary *Mtb* infection in mice. Numerous previous studies demonstrated that TNF and/or TNF-RI expression in hematopoietic cells is absolutely required for the host resistance to *Mtb*. Indeed, WT mice transplanted with bone marrow from *Tnf*<sup>−/−</sup> or *Tnfr1*<sup>−/−</sup> mice were shown to be extremely susceptible to *Mtb* infection and succumb in 4 weeks. We report here that TNF from and TNF-RI on stromal cells are also required to mediate optimal...
host resistance to *Mtb*. Although TNF-RI-signaling is pivotal for protective immunity against *Mtb* (Flynn et al., 1995), abundant TNF expression in the lungs of IL-1RI-deficient mice fails to provide protection and *Il1r1*<sup>−/−</sup> mice also succumb to *Mtb* infection within 4 weeks (Fremond et al., 2007; Sugawara et al., 2001). Collectively, these results obtained with mice deficient in individual components of distinct molecular pathways indicate that these cytokines cannot fully compensate for the lack of one another, thus highlighting their non-redundant and synergistic roles in mounting effective protection from *Mtb*.

It was recently proposed that the mechanism of IL-1-dependent control of *Mtb* is related to COX2-dependent synthesis of prostaglandin E2 (PGE2), which, in turn, suppresses IFN-I that has pro-pathogenic properties (Mayer-Barber et al., 2014). However, earlier studies on leukocyte dynamics in the lungs of mice at early time points after *Mtb* infection show that the likely pro-pathogenic function of IFN-I depends on its ability to recruit *Mtb*-susceptible monocyte subsets to the lungs (Desvignes et al., 2012). Furthermore, *Ifnar1*<sup>−/−</sup> mice had lower pathogen burden in the lungs 18 days p.i. and demonstrated no difference in bacterial burden at 24 days p.i., compared to WT mice (Desvignes et al., 2012). These data suggest that IFN-I signaling is likely to be dispensable for early cell-intrinsic pathogen control and unlikely to be relevant to mechanisms allowing for exacerbated replication of *Mtb* in the lungs of *Il1r1*<sup>−/−</sup> mice that succumb by 4 weeks p.i.

Here we found that IL-1 and TNF signaling to and from the hematopoietic and stromal compartments cooperated to control early *Mtb* infection. When hematopoietic cells lacked IL-1RI signaling, the mice were severely susceptible to *Mtb* infection, and the presence of TNF could not compensate for this deficiency. Our data show that when IL-1RI is completely lacking, a population of *bona fide* neutrophils with CD11b<sup>+</sup>CD11c<sup>−</sup>Ly6G<sup>high</sup> markers in *Mtb* infected mice fail to upregulate TNF-RI and produce ROS, leading to significantly higher bacterial burden and higher necrotic death in vivo, thus suggesting that this specific population of cells may require IL-1RI-dependent licensing to upregulate TNF-RI and induce ROS production in vivo. The excessive *Mtb* replication and necrotic death led to hyper inflammation and the recruitment to the lungs of CD64<sup>+</sup>CD11b<sup>+</sup>Ly6G<sup>low</sup> monocytes, which were highly permissive to *Mtb* irrespective of high amounts of TNF-RI or ROS production. The population of highly *Mtb* permissive CCR2<sup>+</sup> monocytes as propagators of mycobacterial infection has been recently described and is consistent with our findings (Cambier et al., 2014; Lyadova et al., 2010). We further found that the exposure of immortalized mouse lung epithelial cells to a combination of IL-1β and TNF or heat-killed *Mtb* and TNF resulted in a robust up-regulation of TNF and IL-1α expression in these non-hematopoietic cells. It is noteworthy that although the lack of either TNF-RI or IL-1RI on stromal cells allowed for relatively long survival of mice, the lack of both of these receptors simultaneously on stromal cells resulted in the collapse of host resistance despite of the presence of both receptors on WT hematopoietic cells. Taken together, these data provide direct evidence for non-redundant and synergistic roles of IL-1RI- and TNF-RI-dependent signaling in mediating cross-talk between stroma and hematopoietic cells that is necessary for the optimal control of *Mtb* infection.

Our study also revealed that IL-1α, a key cytokine implicated in driving host responses to cell damage and sterile inflammation (Chen et al., 2007), plays a critical and non-redundant
role in host protection against pulmonary Mtb in a non-cell intrinsic manner. The Il1a−/− mice succumb consistently earlier than Il1b−/− mice upon infection with different virulence Mtb strains. Although TNF signaling was earlier implicated in maintaining M.marinum granuloma structure in zebrafish model, TNF-signaling is not required for granuloma formation (Clay et al., 2008). During chronic Mtb infection of Il1a−/− mice, we observed large diffuse inflammatory lesions and dispersed distribution of Mtb-infected cells, suggesting an important role of IL-1α-driven cell-cell cross-talk for coordinating protective granuloma formation or maintenance. IL-1α can function as a plasma membrane-bound cytokine (Dinarello, 2009; Huleihel et al., 1990; Kurtjones et al., 1985; Niki et al., 2004). Because granuloma formation is principally a focal inflammatory response to Mtb infection, the key role of IL-1α in driving cell-cell cross-talk may be an essential function of this cytokine to ensure host protection and pathogen control. In this context, although our study provides direct evidence that IL-1α-deficient mice failed to control Mtb replication at the level of individual infected cells, this defect manifests itself only in vivo and is not cell intrinsic. Indeed, Mtb bacilli replicated in vitro with identical efficacy in bone marrow-derived macrophages harvested from either WT or Il1a−/− mice. Our findings comparing Il1a−/− and Il1b−/− mice seem to contradict previously reported data, which demonstrate that IL-1β-deficient mice are highly susceptible to Mtb and died within 4 weeks post infection, similar to IL-1RI-deficient mice (Mayer-Barber et al., 2010). In our study and the study reported by Mayer-Barber, the specific inactivating mutations of the Il1b gene are distinct (Horai et al., 1998; Shornick et al., 1996) and may explain the discrepancy between our findings.

Clearly, understanding the mechanistic role of IL-1-and TNF driven cell-cell crosstalk in triggering innate mechanisms of host resistance to Mtb can provide the rationale for the development of approaches and drugs to limit the incidence of both progressive and latent tuberculosis through modulating pathways of innate immunity.

**Experimental procedures**

**Experimental Animals**

All studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee guidelines of the University of Washington and Seattle Biomedical Research Institute where all the work involving animals was conducted. C57BL/6 mice were purchased from Charles River, Wilmington, MA. Il1r1−/− and Tnf−/− mice were purchased from Jackson Laboratory. Casp1−/− Il1r1−/− mice were kindly provided by Dr. R. Flavell (Yale University) and described in (Kuida et al., 1995); Il1a−/−, Il1ab−/− mice were described in (Horai et al., 1998). Il1b−/− mice were described in (Shornick et al., 1996). All mice were on C57BL/6 genetic background, matched by age and housed in specific-pathogen-free facilities.

**Bacterial Infection**

Stock of Mtb strains H37Rv, H37Rv::pBP10 (Gill et al., 2009), Erdman, or W-Beijing SA161 were sonicated before use, and mice were infected in an aerosol infection chamber.
To determine the *Mtb* burden, at indicated times the left lung of each mouse was homogenized in PBS with 0.05% Tween 80. Ten-fold serial dilutions were made in PBS with 0.05% Tween 80 and plated on Mitchison 7H10 plates. Colonies were counted after 21 d of incubation at 37°C, and CFUs per organ were determined. Approximately 100 CFUs were deposited in the lungs of each mouse upon initial infection. Mice were sacrificed when the body weight declined by 20%, compared to the body weight of each animal at the day of infection.

**Histology and immunofluorescence staining**

For hematoxylin and eosin, acid-fast Kinyoun’s, and electron microscopy studies, the right lung was excised, fixed in 4% paraformaldehyde (PFA) for 1 week at room temperature, embedded in paraffin, and sectioned by microtome in consecutive sections of 5 µm. For confocal immunofluorescence, staining was performed on frozen sections. For PI analyses, dry frozen sections were evaluated under a fluorescent microscope without further processing.

**Cell Isolation, Staining and Flow Cytometry**

Briefly, single-cell suspensions of intraparenchymal lung lymphocytes were stained at saturating conditions using antibodies specific for CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD11c (HL), CD11b (M1/70), Gr-1 (RB6-8C5), CD45.2 (104), CD45.1 (A20), Ly-6C (HK1.4), Ly-6G (1A8), CD40 (1C10) all from BD Biosciences. IFN-γ and TNF intracellular staining was performed using a kit as instructed by the manufacturer (BD), with minor modifications. For direct ex-vivo detection of IFN-γ, lung cells were isolated in the presence of Brefeldin A (Sigma) and staining for surface markers and intracellular IFN-γ was performed as described, without in vitro restimulation. A minimum of 100,000 live cells per sample was acquired on an LSRII instrument using the FACSDiva software (BD Biosciences). The samples were then analyzed by Flowjo Software (Tree Star, Inc.). TNF-RI and mCherry analyses were done using CD64 and CD11c-gating approach (Figure S1A). ROS staining was analyzed using alternate CD11b and CD11c-gating approach (Figure S1D) to avoid spectral overlap between ROX reagent and CD64 Ab staining.

**Detection of ESAT-64–17– and TB10.44–11–specific cells**

PE-labeled MHC class II tetramers (I-A\(^b\)) containing the stimulatory residues 4 to 17 (QQWNFAGIEAAASA) of the early secreted antigenic target 6 kD (ESAT-6) of *Mtb* and APC-labeled MHC class I tetramers (K\(^b\)) containing the stimulatory residues 4 to 11 (IMYNYPAM) of the low molecular weight protein antigen TB10.4 of *Mtb* (obtained from the National Institutes of Health Tetramer Core Facility) were used to detect *Mtb*-specific CD4\(^+\) and CD8\(^+\) T cells, respectively.

**Statistical Analysis**

All experiments were repeated at least twice and a minimum number of samples analyzed per each experimental group was equal to three or more. Results are expressed as mean and standard error. Unless otherwise indicated, Student’s unpaired two-tailed t test was used for comparing experimental groups, with *P* < 0.05 considered significant.
Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. IL-1RI signaling on hematopoietic cells is required for host resistance to Mtb
(A) Survival of indicated bone marrow chimeric, gene deficient, and WT mice after infection with Mtb. N = 10 for WT, N = 10 for WT-WT, N = 12 for Il1r1−/−-WT, N = 8 for Il1r1−/−, N = 6 for Il1r1−/−-Il1r1−/−; N = 20 for Il1ab−/− mice. Experimental groups were compared with the log rank test to WT or WT-WT groups. **P < 0.01.
(B) Expression of cytokines and chemokines in the lungs of mice deficient for indicated genes or WT mice infected with Mtb 30 days p.i. determined by Proteome Profiler mouse Immunity. Author manuscript; available in PMC 2016 December 15.
cytokine antibody array. N=4. Pos-C are dots that show the manufacturer’s internal positive control samples on the membrane. WT-mock: the sample of a mouse lung without infection.

(C) Percentage of Mtb-infected mCherry-positive cells in the lungs of WT and Il1r1−/− mice 17 days p.i.

(D) Dot plots of cells isolated from the lungs of Il1r1−/− and WT mice after infection with H37Rv and mCherry-expressing H37Rv Mtb strains 17 days p.i. with gating defining autofluorescent and non-autofluorescent populations. The distribution of autofluorescent mCherry+ cells on CD11c+ and CD64+ populations is shown. N=4.

(E) Images of representative non-autofluorescent (488-Auto−) and autofluorescent (488-Auto+) cells with indicated markers isolated from the lungs of WT and Il1r1−/− mice 17 days p.i., collected with Flow Imager camera.

(F) Percentage of Mtb-infected cells with indicated phenotypes in the lungs of WT and Il1r1−/− mice 17 days p.i. N=4. **P < 0.01.

(G) Percentage of Mtb-infected Ly6Ghigh and Ly6Glow cell population in the lungs of WT and Il1r1−/− mice 17 days p.i. N=4. *P<0.05. ***P<0.001.

(H–J) Expression of TNF-RI, ROS, and mCherry signal on Mtb-infected 488-Auto+ and 488-Auto− Ly6Ghigh cells. N=4. *P<0.05. **P<0.01. ***P<0.001.

(K–M) Expression of TNF-RI, ROS, and mCherry signal on Mtb-infected 488-Auto+ and 488-Auto− Ly6Glow cells. N=4. *P<0.05. **P<0.01. ***P<0.001.

(I) Macroscopic evaluation of Mtb bacilli distribution on the sections of lungs isolated from WT and Il1r1−/− mice 30 days p.i. and stained by acid fast staining. Representative fields are shown. N=5. For extended data in Figure 1, see also Figure S1.
Figure 2. Effective control of pulmonary *Mtb* infection is enabled through IL-1–TNF-dependent cytokine-stroma cross-talk

(A) Survival of indicated bone marrow chimeric mice after infection with *Mtb*. N=5 to 7 per experimental group. Experimental groups were compared to control WT-WT group with the log rank test. **P < 0.001; median survival in days p.i. for groups is shown (Med.survival); n.d. – not defined prior to termination of the experiment.

(B–C) Expression of indicated cytokines and chemokines in primary bone marrow macrophages (B) or immortalized mouse lung cells (MLg) (C) after their treatment with

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IL-1β, TNF, heat-killed Mtb (H.k. Mtb) or their combinations. Cells were analyzed 24 h after treatment with Proteome Profiler mouse cytokine antibody array. N=3. Pos-C are dots that show the manufacturer’s internal positive control samples on the membrane. Control cells were treated with media only (Mock).

(D) Expression of IL-1α on the surface of bone marrow macrophages after their treatment with LPS (50 ng/ml) or heat-killed Mtb 4 hours after stimulation and determined by flow cytometry.

(E) Production of CXCL1 chemokine by mouse lung epithelial cell line LA4 after treatment with recombinant mouse IL-1α with or without prior incubation of cells with anti-IL-1α monoclonal Ab (MAb). The amount of CXCL1 was determined by ELISA. N=4. *P<0.01.

(F) Production of CXCL1 chemokine by LA4 cells after their co-culture with LPS-treated bone marrow macrophages from WT, Casp1−/−, or Casp1−/−Il1a−/− mice stimulated to produce membrane-bound IL-1α (mem-IL-1α) with or without addition of anti-IL-1α MAb. N=4; *P<0.01.

(G) Expression of indicated cytokines and chemokines in MLg cells after their co-culture with LPS-treated bone marrow macrophages from Casp1−/− or Casp1−/−Il1a−/− mice, stimulated to produce membrane-bound IL-1α (C1-memIL-1α or C1.II1a-memIL-1α, as a negative control) with or without the addition of TNF. Cells were analyzed 24 h after treatment with Proteome Profiler mouse cytokine antibody array. N=3. Pos-C are dots that show the manufacturer’s internal positive control samples on the membrane. Control cells were treated with media only (Mock).

(H) Survival of indicated bone marrow chimeric mice after infection with Mtb. N=5 to 7 per experimental group. Experimental groups were compared to control WT-WT group with the log rank test. **P < 0.001; median survival in days p.i. for groups is shown (Med.survival); n.d. – not defined prior to termination of the experiment.
Figure 3. IL-1α and IL-1β play distinct roles in mediating long-term resistance to Mtb

(A) Survival of indicated gene-deficient mice after infection with Mtb. N=12 for WT; N=14 for Il1r1−/−; N= 13 for Il1ab−/−; N= 12 for Il1a−/− and N= 9 for Il1b−/− mice. **P < 0.01.

(B) Bacterial loads in the lungs of Mtb-infected mice as evaluated by plating serial dilutions of lung homogenates on 7H10 agar. Data are representative of three independent experiments with five mice per experimental group per time point. *P < 0.01.

(C) Lung histopathology in WT and Il1a−/− at 60 days after infection with Mtb analyzed after hematoxylin and eosin (H&E) staining (left panels). Right panels show lung sections stained with acid-fast staining with Mtb bacilli indicated by arrows. The representative images after H&E (left) and acid fast (right) staining are shown. Scale bar is 50 µm.

(D) Quantitative representation of the lung histopathology in WT, Il1a−/−, Il1b−/−, and Il1ab−/− mice at 30, 60, and 90 days after infection with Mtb analyzed after hematoxylin and eosin (H&E) staining using histopathology score scale shown in Figure S2D. Four
consecutive sections of the lungs at three depth levels per each mouse were evaluated. The histopathology score was averaged for 5 mice per each experimental group per time point. *P < 0.05.

(E) Quantitative representation of the inflammation-free airway space in the lungs of WT, Il1a−/−, Il1b−/−, and Il1ab−/− mice at 30, 60, and 90 days after infection with Mtb analyzed after hematoxylin and eosin (H&E) staining. Four consecutive sections of the lungs at three depth levels per each mouse were evaluated. The data was averaged for 5 mice per each experimental group per time point. * P < 0.05. For extended data in Figure 3, see also Figure S2.
Figure 4. Mice deficient in IL-1RI ligands develop functional pathogen-specific adaptive immunity

(A) Representative dot-plots showing the proportion of IFN-γ-producing cells in populations of ESAT6<sup>4-17</sup>-I-A<sub>b</sub>-specific CD4<sup>+</sup> T cells purified from the lungs of WT, II1a<sup>-/-</sup>, and II1ab<sup>-/-</sup> mice at 30 days after Mtb infection. N = 4.

(B) Quantitative representation of data shown in (A). * P < 0.001. n.s. – not significant.
(C) Representative dot plots showing the proportion of IFN-γ-producing cells in populations of TB10.4+;Kb-specific CD8+ T cells purified from the lungs of WT, Il1a−/−, and Il1ab−/− mice at 30 days after Mtb infection. N = 4.

(D) Quantitative representation of data shown in (C). *p < 0.001. n.s. – not significant.

(E) Flow cytometry analysis of INF-γ and TNF expression by CD4+ and CD8+ T cells purified from the lungs of WT or Il1a−/− mice 60 days after infection with Mtb and re-stimulated with indicated stimuli in vitro. Purified cells were stimulated with the media only (Mock), a mixture of anti-CD3 and anti-CD28 antibodies, or synthetic ESAT6 (ESAT-64–17 peptide) or TB10.4 (TB10.44–11 peptide)–specific peptides respectively. Cumulative data showing the average percentages of lung CD4+ or CD8+ T cells producing IFN-γ and TNF in response to indicated stimuli in individual mice (three mice per group) are shown. No statistically significant differences in in vitro responses to analyzed stimuli between cells purified from WT and Il1a−/− mice were found. For extended data in Figure 4, see also Figure S3.
Figure 5. The IL-1α-deficient mice develop non-resolving monocytic inflammation

(A) The weight of lungs harvested from WT and Il1a−/− mice at 90 days after Mtb infection. N = 30, ** P < 0.01.

(B) Total number of mononuclear cells purified from lungs of WT are Il1a−/− mice at indicated times after Mtb infection. N = 5. * P < 0.05; ** P < 0.01.

(C) Flow cytometry dot plots of the distribution of Ly-6C- and Ly-6G-expressing cell subsets in the lungs of WT and Il1a−/− mice 90 days after Mtb infection. The average percentage of Ly-6C+ or Ly-6C+Ly-6G+ populations are shown for each strain. N=4.

(D) Analysis of Ly-6C expression on CD11b- and CD11c-expressing subsets of mononuclear cells purified from the lungs of Mtb-infected WT and Il1a−/− mice. Median intensity of Ly-6C staining on CD11b+ and CD11c+ (population b) subsets are shown in histogram plots on the right. N=4. a and b designate individual Ly-6C+ cell populations expressing different amounts of CD11c.

(E) Analysis of CD40-expressing CD11c+ cell subsets in the lungs of WT and Il1a−/− mice after Mtb infection. The average percentage of CD11c+CD40high (population a) and CD11c+highCD40low (population b) subset are shown. N=4.

(F) Flow cytometry dot plots of CD11c+CD40+ cells in the lungs of bone marrow chimeric WT mice, transplanted with either WT or Il1a−/− bone marrow cells. Recipient WT mice were lethally irradiated and transplanted with bone marrow cells from either WT or Il1a−/− mice. Five months after the bone marrow transplantation, mice were infected with Mtb and
indicated cell subsets were analyzed in the lungs 80 days after infection. Average percentage of CD11c<sup>+</sup>CD40<sub>high</sub> and CD11c<sub>high</sub>CD40<sub>low</sub> are shown. N=3.
Figure 6. Restoration of IL-1α expression in CD11c<sup>+</sup> cells alleviates pulmonary *Mtb* susceptibility phenotype of IL-1α-deficient mice

(A) Confocal microscopy analysis of IL-1α expression in *Mtb* infected cells of lung granulomas 60 days after infection on frozen OCT-embedded sections of lungs. IL-1α-specific antibody staining was developed with Cy2-labeled secondary antibody (green). *Mtb* antigens were stained with polyclonal primary antibody and developed with Cy3-labeled secondary antibody (red). *Mtb*-infected cells stained positive with IL-1α antibody are indicated by arrows. Bar is 20µm. Confocal images were obtained using a Zeiss 510 Meta Confocal microscope. Representative fields are shown. N = 4.
(B) Confocal microscopy analysis of IL-1α expression in cells of lung granulomas 60 days after infection on frozen OCT-embedded sections of lungs. IL-1α-specific antibody staining was developed with Cy2-labeled secondary antibody (green). CD11b, CD11c, CD3, and Gr1 antigens were stained with rat monoclonal primary antibody and developed with Cy3-labeled secondary antibody (red). Bar is 20 µm. Representative fields are shown. N = 4.

(C–D) Analysis of CD40-expressing CD11c+ cell subsets in the lungs of Il1a−/− mice transplanted with foamy-virus-infected hematopoietic stem cells 60 days after Mtb infection. The average percentages of CD11c+CD40high and CD11c+CD40low subset are shown.

(E) Survival of mice transplanted with CD11c-IL-1α- or CD11c-stop-IL-1α- foamy virus-transduced hematopoietic stem cells after their infection with Mtb. N = 10 for each experimental group. ** P < 0.001. Groups were compared with a log rank test. For extended data in Figure 6, see also Figure S4.
Figure 7. IL-1α is required for intracellular control of Mtb replication in vivo

(A) The number of Mtb bacilli on acid fast-stained sections of mouse lungs at indicated time point. The numbers of bacilli were counted for seven view fields at four tissue depth levels per mouse and were averaged for five mice per experimental group per time point. * P < 0.05, ** P < 0.01.

(B) Representative fields of granulomas with Mtb-infected cells (depicted by stars) in the lungs of WT and Il1a−/− mice at 60 days p.i. Lung sections were stained with acid fast to visualize Mtb bacilli (pink).

(C) Quantitation of cells containing less than- or over four Mtb bacilli on the sections of lungs from WT or Il1a−/− mice 60 days p.i. ** P < 0.01. Cells were quantified on lung sections as described in (A).

(D) Electron microscopy analysis of cells infected with Mtb in the lungs of Il1a−/− mice 60 days p.i. Left panel – original magnification 5,000× right panel shows selected area of an infected cell at magnification of 30,000×. Representative cell is shown. N > 50.

(E) Mtb replication rate between days 1 and 11 in the lungs of WT and Il1a−/− mice determined based on pBP10 plasmid frequency and previously described mathematical model (Gill et al., 2009). ** P < 0.01. For extended data in Figure 7 and the model, see also Figures S5 and S6.