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TPL-2–ERK1/2 Signaling Promotes Host Resistance against Intracellular Bacterial Infection by Negative Regulation of Type I IFN Production

Finlay W. McNab,*†1 John Ewbank,*†1 Ricardo Rajsbaum,*†† Evangelos Stavropoulos,* Anna Martirosyan,*, Paul S. Redford,* Xuemei Wu,*, Christine M. Graham,*, Margarida Saraiva,†‡§ Philip Tschijis,¶ Damien Chaussabel,†# Steven C. Ley,** and Anne O’Garra*†††

Tuberculosis, caused by Mycobacterium tuberculosis, remains a leading cause of mortality and morbidity worldwide, causing ∼1.4 million deaths per year. Key immune components for host protection during tuberculosis include the cytokines IL-12, IL-1, and TNF-α, as well as IFN-γ and CD4+ Th1 cells. However, immune factors determining whether individuals control infection or progress to active tuberculosis are incompletely understood. Excess amounts of type I IFN have been linked to exacerbated disease during tuberculosis in mouse models and to active disease in patients, suggesting tight regulation of this family of cytokines is critical to host resistance. In addition, the immunosuppressive cytokine IL-10 is known to inhibit the immune response to M. tuberculosis in murine models through the negative regulation of key proinflammatory cytokines and the subsequent Th1 response. We show in this study, using a combination of transcriptomic analysis, genetics, and pharmacological inhibitors, that the TPL-2–ERK1/2 signaling pathway is important in mediating host resistance to tuberculosis through negative regulation of type I IFN production. The TPL-2–ERK1/2 signaling pathway regulated production by macrophages of several cytokines important in the immune response to M. tuberculosis as well as regulating induction of a large number of additional genes, many in a type I IFN-dependent manner. In the absence of TPL-2 in vivo, excess type I IFN promoted IL-10 production and exacerbated disease. These findings describe an important regulatory mechanism for controlling tuberculosis and reveal mechanisms by which type I IFN may promote susceptibility to this important disease. The Journal of Immunology, 2013, 191: 1732–1743.
Iffnr1−/− mice have greatly reduced bacterial loads following L. monocytogenes infection (30–33). A number of mechanisms have been suggested for the exacerbated disease induced by type I IFN during L. monocytogenes infection, including induction of IL-10 following T cell apoptosis and downregulation of the IFN-γ receptor (30–34).

Results from M. tuberculosis infection of Iffnr1−/− mice have varied between studies (35–40), but overall suggest a detrimental role for type I IFN during tuberculosis (37–39). Increased levels of type I IFN were induced by infection of mice with several hyperpervurilant strains of M. tuberculosis and resulted in exacerbated disease (36, 37). Furthermore, in mouse models in which large amounts of type I IFN were induced following administration of the polynosinic-polycytidylic acid derivative Poly-ICLC during M. tuberculosis infection, tuberculosis was more severe (40). Direct instillation of IFN-αβ into the lung of M. tuberculosis–infected mice also resulted in exacerbated disease (36). We have recently reported a potential role of type I IFN in human tuberculosis, because active tuberculosis patients showed a prominent type I IFN-inducible gene signature in their blood, which correlated with extent of radiotherapy. M. tuberculosis patients showed a prominent type I IFN-inducible gene signature following L. monocytogenes infection (41).

The mechanisms by which type I IFN exacerbates tuberculosis are less clear than for L. monocytogenes infection; however, a recent report has shown that type I IFN suppresses the host-protective cytokine IL-1, an effect that was partially due to IL-10 (20). IL-10 is a broadly immune-suppressive cytokine produced by cells of both the innate and adaptive immune systems (42) that can suppress the host-protective immune response during M. tuberculosis infection (43–45) and L. monocytogenes (46–48) infection.

The regulation of cytokine production by macrophages and other innate cells during M. tuberculosis infection is likely to shape the downstream immune response and determine disease outcome. Pattern recognition receptors (PRR), such as TLR, expressed by innate immune cells, are critical in the activation of these cells and induction of cytokines in response to infection (49). Signaling pathways triggered by these PRR will vary depending on the MAP3K tumor pyruvate, IFN-β was obtained from PBL, and IFN-γ from R&D Systems. DMSO was from Sigma-Aldrich. Generation and infection of bone marrow–derived macrophages and sorting and infection of bone marrow monocytes

Bone marrow cells were flushed from the femurs and tibia of mice and plated at 0.5 × 10^6 cells/ml on bacterial plates (Sterilin), in culture medium containing 10% FCS and 20% L929 cell-conditioned medium. At day 6, macrophages were harvested and seeded into 24-well tissue culture plates (Corning) at 1 × 10^5 cells/ml in culture medium. Cells were rested overnight, washed once with PBS, and infected at multiplicity of infection (MOI) 2:1 with M. tuberculosis H37Rv. Where indicated, IFN-β (2 ng/ml) was added concomitantly with M. tuberculosis H37Rv. For inhibitor experiments, cells were pretreated for 30 min with 0.1 μM of either the MEK1/2 inhibitor PD0325901 or DMSO vehicle control. A total of 0.1 μM PD0325901 was chosen based on data presented in Bain et al. (65) and on a detailed titration of the inhibitor on bone marrow–derived macrophage, done for this study (data not shown), to determine a dose that gave robust inhibition, but was low enough to avoid toxicity or potential off-target effects. For L. monocytogenes infection, macrophages were generated as described above and infected with L. monocytogenes at a MOI of 1:1. At 1 h postinfection, cells were washed once with PBS, and media containing 10 μg/ml gentamicin added to inhibit growth of extracellular bacteria. Where indicated, IFN-α or IFN-β (both 2 ng/ml) was added concomitantly with L. monocytogenes.

Bone marrow monocytes were isolated by FACS on a Beckman-Coulter MoFlo. Bone marrow cell suspensions were stained with Abs against CD3, CD19, CD11c, F4/80, Ly6G, and Ly6C, purchased from eBioscience. Monocytes were sorted as CD3−CD19−CD11c−F4/80−Ly6G−Ly6C+. Cells were then plated at 1 × 10^6 cells/ml and infected with H37Rv at MOI 2:1.

Measurement of supernatant and serum cytokines

Cytokine concentrations in the supernatant of infected cells were determined using commercial kits for IFN-β and IFN-α (PBL), TNF-α and IL-12p70 (eBioscience), and IL-1β (R&D Systems). IL-12p40 was detected using Ab clone C15.6.7 for capture and biotinylated Abs clone C17.8 for detection. IL-10 was detected using Ab clone JES5-2A5 for capture and biotinylated anti–IL-10 for detection (BD Biosciences). Custom magnetic bead arrays to measure IL-10, IL-12p40, IL-12p53, and IFN-γ in serum were purchased from Millipore and used according to manufacturer’s instructions. Samples were run on a Bio-Rad Lumex200 machine.

Processing of macrophage RNA for quantitative PCR and microarray analysis

At indicated times postinfection, supernatants were removed and cells were washed once with PBS. RNA was harvested in 350 μL RLT buffer (Qiagen) and stored at −80 °C before processing. RNA was processed using RNasy mini-kits (Qiagen). RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). Samples had a RNA integrity number of 9.3–10.
For microarray analysis, biotinylated, amplified antisense cRNA was prepared from 300 ng total RNA using the Illumina TotalPrep RNA amplification kit (Applied Biosystems/Ambion). A total of 750 ng labeled cRNA was hybridized overnight to Illumina Mouse WG-6 V2.0 Beadchip arrays (Illumina). The arrays were then washed, blocked, stained, and scanned on an Illumina BeadStation 500 following the manufacturer’s protocols. Illumina BeadStudio software (Illumina) was used to generate signal intensity values from the scans. For quantitative PCR, RNA was reverse-transcribed with a high-capacity reverse-transcription kit (Applied Biosystems) to cDNA. The expression of indicated genes was quantified by real-time PCR (ABI PRISM 7900; Applied Biosystems) and normalized against Hprt1 mRNA levels. Murine primers were all purchased from Applied Biosystems.

Microarray data analysis
Illumina BeadStudio software was used to subtract background from signal intensity values. GeneSpring GX version 11 (Agilent Technologies) was used to perform further analysis. All signal intensity values < 10 were set to equal 10. This threshold step is a standard analysis step done for Illumina microarray data. Due to the lower sensitivity of the lower intensity values obtained from Illumina microarray data, it is necessary to reduce type 1 error (false positives) occurring when applying fold change data. Data were then normalized by shifting to the 75th percentile. Per-gene normalization was then applied by dividing the signal intensity of each probe in each sample by the median intensity for that specific probe, which is obtained across all samples. This is also a standard step for Illumina microarray data. Because this calculation is done in the log scale, it acts as a subtraction. This step is performed to reduce nonbiological differences, and, although it may affect the absolute values, it does not affect the fold change between values: the measurement used in this study.

All transcripts were filtered first to select detected transcripts: detected transcripts were defined as those present (p < 0.01) in 100% of triplicate samples within any one group. Present calls were selected if the signal precision (a probe’s intensity value compared with the background intensity value) was statistically significantly different (p < 0.01). Statistical and fold-change analyses were then applied, as described in the figure legends, to generate lists of differentially expressed genes. These were subjected to hierarchical clustering, using Pearson centered distance metric and complete linkage, and visualized using a heat map. Microarray data have been deposited at the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/), accession number GSE47674.

Statistics
Statistical tests, as described in the figure legends, were used to determine significance. *p < 0.05, **p < 0.01, ***p < 0.001.

Results
The TPL-2–ERK1/2 pathway is rapidly activated in macrophages infected with M. tuberculosis
To investigate the transcriptional regulation of cytokine production in response to M. tuberculosis infection, we infected bone marrow–derived macrophages with M. tuberculosis and carried out microarray analysis of purified RNA from cells harvested at various times postinfection. Removal of undetectable genes and those that did not show a > 2-fold change upon infection, together with statistical filtering, resulted in 6479 differentially regulated transcripts, which were then subjected to supervised hierarchical clustering (Fig. 1A). We used k-means clustering to separate these 6479 transcripts into discrete clusters of genes that had similar expression kinetics (Supplemental Fig. 1). The clusters showed a range of expression patterns, with some induced rapidly (between 30 min and 1 h postinfection) and others expressed relatively late (between 6 and 24 h postinfection) (Supplemental Fig. 1). Two clusters in particular were upregulated between 30 min and 1 h postinfection. The first remained elevated throughout the course of infection and contained several proinflammatory cytokines and chemokines (cluster 5, Suplemental Fig. 1). In contrast, the genes in the second early cluster (cluster 15, Supplemental Fig. 1) were only transiently upregulated, with most genes returning to baseline by 3–6 h postinfection (Fig. 1B, 1C). Interestingly, many of the genes in this cluster have previously been described as targets of the ERK1/2 MAPK pathway, including Fos (54, 62, 63), Egr1 (62, 63), and Dusp1 (64) (Fig. 1C, 1D).

TPL-2 negatively regulates IL-12 and IFN-β, but positively regulates IL-10, IL-1β, and TNF-α, in M. tuberculosis–infected macrophages and monocytes
The TPL-2–ERK MAPK pathway has previously been shown to be a key regulator of cytokine production in innate immune cells, in response to a variety of TLR ligands, including LPS, Pam-3-Cys, and CpG (51, 52, 54, 55). We next investigated whether this pathway also regulated cytokines similarly in response to M. tuberculosis infection, which activates multiple PRRs, including both TLR and non-TLR (4, 5). Addition of PD0325901, which specifically inhibits the catalytic activity of the MAP2K MEK1/2 and abrogates subsequent ERK activation (65), resulted in increased production of IL-12p40, IL-12p70, and IFN-β and decreased production of IL-10, TNF-α, and IL-1β, in M. tuberculosis–infected macrophages (Fig. 2A). This effect was phenocopied in Tpl2−/− macrophages, which secreted markedly increased levels of IL-12p40, IL-12p70, and IFN-β, but less IL-10, TNF-α, and IL-1β, in response to M. tuberculosis infection, as compared with WT controls (Fig. 2B). The effects of TPL-2 deficiency on cytokine production following M. tuberculosis infection, therefore, appeared to result from impaired ERK activation, similar to previous studies using purified TLR ligands (51, 52, 54, 55). Finally, to confirm that similar responses were seen in ex vivo derived cells, monocytes were sorted from the bone marrow of WT and Tpl2−/− mice and infected with M. tuberculosis. The phenotype of these cells (Fig. 2C) mirrored that seen using Tpl2−/− macrophages, or following MEK1/2 inhibition.

The TPL-2–ERK1/2 pathway is an important regulator of transcription in M. tuberculosis–infected macrophages
Having shown that the TPL-2–ERK1/2 pathway regulates cytokine protein production in M. tuberculosis–infected macrophages, we next carried out microarray analysis of RNA from infected WT and Tpl2−/− macrophages, to determine in an unbiased fashion how the TPL-2–ERK pathway regulates gene expression at a global level. We chose 6 h postinfection for this analysis, as this was the peak of transcription of the majority of genes observed in WT macrophages infected with M. tuberculosis (Fig. 1A).

To determine which M. tuberculosis–induced genes were significantly differentially regulated in WT versus Tpl2−/− macrophages, we carried out fold-change analysis of genes that were over- or underexpressed upon M. tuberculosis infection of macrophages to first determine those genes regulated by M. tuberculosis infection. This set of M. tuberculosis–regulated genes was then further analyzed by statistical filtering and fold-change analyses, now comparing the WT and Tpl2−/− macrophages, to determine which of the M. tuberculosis–regulated genes were significantly regulated by TPL-2 upon M. tuberculosis infection. This identified 104 differentially expressed genes (Fig. 3A), containing a prominent cluster of genes that were upregulated to a greater extent in Tpl2−/− macrophages (Fig. 3A). Genes present in this cluster included the class-2 transactivator (C2a), plasmin (Plat), and chloride intracellular ion channel 5 (Clc5). Importantly, Il2a (encoding the p35 subunit of IL-12) and several members of the type I IFN family, including Ifna2, Ifna5, and Ifna6, were also found in this cluster of elevated genes (Fig. 3A).
A second cluster contained genes that were downregulated to a greater extent in Tpl2−/− macrophages, including the genes encoding Arginase (Arg1) and dual specificity phosphatase 6 (Dusp6) (Fig. 3A).
Interestingly, this downregulated cluster of genes also included that encoding a subunit of the IFN-γ receptor (Ifngr1), which is
crucial in activation of myeloid cells for resistance to \textit{M. tuberculosis} infection (Fig. 3A). We confirmed the elevated expression levels of \textit{Il12a}, \textit{Ifna2}, \textit{Ifna5}, \textit{Ifna6} mRNAs and reduced expression of \textit{Ifngr1} mRNA by quantitative RT-PCR (qRT-PCR) of RNA taken from the same \textit{M. tuberculosis}–infected \textit{Tpl}2\textsuperscript{2/2} and WT control macrophages used for microarray (Fig. 3B). Although not detected in the microarray, qRT-PCR for \textit{Il12b} mRNA (encoding the IL-12p40 subunit) revealed elevated expression levels in \textit{Tpl}2\textsuperscript{2/2} macrophages (Supplemental Fig. 3), similarly to the results seen for \textit{Il12a}. A third cluster of differentially regulated transcripts was also found, which contained the immediate early genes, \textit{Ier3} and \textit{Ier5} (Fig. 3A).

The list of 104 differentially expressed genes shown by microarray did not contain IFN-\(\beta\) itself, IL-1\(\beta\), TNF-\(\alpha\), or IL-10, despite differences being observed at the protein level (see Fig. 2) and in contrast to our and others’ previous reports showing these cytokines to be regulated by TPL-2 in macrophages in response to TLR ligands (50–60). However, qRT-PCR revealed that the mRNA encoding IFN-\(\beta\) was actually significantly upregulated in \textit{Tpl}2\textsuperscript{2/2} macrophages compared with WT (Fig. 3B, Supplemental Fig. 3), similarly to the results seen for \textit{Il12a}. A third cluster of differentially regulated transcripts was also found, which contained the immediate early genes, \textit{Ier3} and \textit{Ier5} (Fig. 3A).

\textit{TPL-2-deficient mice are more susceptible to intracellular bacterial infection}

Given the complex regulation by TPL-2–ERK1/2 signaling in \textit{M. tuberculosis}–infected macrophages of cytokines known to be either host protective or favoring chronic infection, we investigated the net effect of abrogation of TPL-2–ERK1/2 signaling during intracellular bacterial infection in vivo. WT and \textit{Tpl}2\textsuperscript{2/2} mice were aerosol infected with \textit{M. tuberculosis} H37Rv, or alternatively i.v. infected with \textit{L. monocytogenes}. Organs from \textit{M. tuberculosis}–infected mice were harvested at days 28, 56, and 100 postinfection for enumeration of bacterial loads (Fig. 4 and data not shown). Although no difference in lung bacterial loads was found early (day 28) following \textit{M. tuberculosis} infection (data not shown), at day 56 \textit{Tpl}2\textsuperscript{2/2} mice had significantly higher bacterial loads in the lung (Fig. 4A) and spleen (data not shown) compared with WT.
mice, with 5- to 10-fold more bacteria in the lung (Fig. 4A). This increase in bacterial load in lungs of Tpl2−/− compared with WT mice was also seen at day 100 postinfection (data not shown). Tpl2−/− mice were also highly susceptible to L. monocytogenes infection, having bacterial loads in the spleen >10-fold higher than WT controls at day 3 postinfection (Fig. 4B). Bacterial loads were also significantly higher in Tpl2−/− mice than WT controls at days 1 and 2 postinfection, although differences were smaller than at day 3 (data not shown).

To determine whether the increased susceptibility of TPL-2–deficient mice involved impaired innate immune responses, WT and Tpl2−/− mice on a Rag1−/− background were infected with M. tuberculosis H37Rv or with L. monocytogenes (Fig. 4A, 4B, bottom rows). Similar to Tpl2−/− versus WT mice, infected Rag1−/−/Tpl2−/− mice had significantly higher bacterial loads compared with their counterpart Rag1−/− controls (Fig. 4A, 4B, bottom rows). These data demonstrate that the increased susceptibility of Tpl2−/− mice could be in part accounted for by a defect in the innate immune response.

TPL-2 negatively regulates expression of type I IFN during bacterial infection

To investigate how TPL-2 deficiency might increase susceptibility of mice to bacterial infection, we measured levels of IL-12, TNF-α, and IL-1β, which are important for protection, and levels of type I IFN and IL-10, known to promote bacterial infection. Cytokine levels in the serum of WT and Tpl2−/− mice infected with L. monocytogenes were measured (Fig. 4C). Levels of IL-12p40 and IL-12p70, IL-1β, and TNF-α in sera of L. monocytogenes–infected mice were not significantly different between groups (Fig. 4C). Although IFN-β was not detectable, strikingly, IFN-α levels were significantly higher in sera from L. monocytogenes–infected Tpl2−/− mice compared with WT (Fig. 4C). Unexpectedly, IL-10 levels were also higher in serum of Tpl2−/− mice, in contrast to the reported requirement for TPL-2–ERK1/2 in endogenous IL-10 production by TLR-stimulated (51, 54, 60) or M. tuberculosis–infected (Fig. 2) innate immune cells in vitro. This suggested that other factors elevated in Tpl2−/−/L. monocytogenes–infected macrophages significantly suppressed IL-10 production by TLR-stimulated (51, 54, 60) or M. tuberculosis–infected (Fig. 2) innate immune cells in vitro. This suggested that other factors elevated in Tpl2−/−/L. monocytogenes–infected macrophages might help to explain the elevated IL-10 levels seen in the serum of Tpl2−/− mice infected in vivo, L. monocytogenes–infected WT or Tpl2−/− macrophages were treated with exogenous IFN-α or IFN-β at the time of infection and IL-10 levels measured (Fig. 4D). Whereas levels of IL-10 overall were, as expected, lower in Tpl2−/− compared with WT macrophages, addition of type I IFNs to Tpl2−/− cells led to a fold increase in IL-10 production equivalent to that seen in WT macrophages treated with type I IFN (Fig. 4D).

Type I IFN signaling regulates transcription in Tpl2−/− macrophages

Given that protein and mRNA levels of type I IFN were greatly increased in in vitro M. tuberculosis–infected TPL-2–deficient macrophages, and that levels of type I IFN were increased in vivo in mice lacking TPL-2, we hypothesized that type I IFN may be contributing to transcriptional changes in innate cells following infection in the absence of TPL-2, leading to increased susceptibility. We analyzed by microarray the expression of the genes previously found to be up- or downregulated by TPL-2 deficiency in M. tuberculosis–infected macrophages (see Fig. 3) in Tpl2−/− or WT macrophages additionally crossed to Ifnar1−/−, to determine whether any TPL-2–regulated genes were strongly regulated
by type I IFN signaling during *M. tuberculosis* infection (Fig. 5). The transcriptional levels of a large number of the genes upregulated in Tpl2−/− macrophages were dramatically lower in Tpl2−/− Ifnar1−/− macrophages, in many cases down to levels observed in Ifnar1−/− control *M. tuberculosis*–infected macrophages (Fig. 5A, genes in bold). These genes included *Il12a*, which intriguingly has a protective role in *M. tuberculosis* infection, but also several subtypes of *Ifna*: *Ifna2*, *Ifna5*, and *Ifna6*; associated with disease exacerbation, as well as *Tapblp*, *Clic5*, *Snn*, and *Hmgn3* (Fig. 5A). This IFNAR-dependent regulation also applied to a cluster of genes that were downregulated by TPL-2 deficiency following infection with *M. tuberculosis*, with many returning to WT levels in Tpl2−/− Ifnar1−/− macrophages (Fig. 5B). These included *Ifngr1*, *Dusp6*, and to a lesser extent *Arg1* (Fig. 5B). We validated the microarray results for *Il12a*, *Ifna2*, *Ifna5*, *Ifna6*, and *Ifngr1* by qRT-PCR, and IL-12p70 by ELISA (Supplemental Fig. 3B, 3C), confirming that the perturbation of expression of these genes/proteins in Tpl2−/− macrophages infected with *M. tuberculosis* resulted from enhanced IFNAR signaling. We also carried out qRT-PCR and ELISA to examine levels of IFN-β, IL-1β, TNF-α, and IL-10 (Supplemental Fig. 3B, 3C). IL-1β transcriptional levels were not rescued in the Tpl2−/− Ifnar1−/− cells, but protein levels were much higher, suggesting posttranscriptional regulation of IL-1β by type I IFN (Supplemental Fig. 3). IL-10 levels, both at the transcriptional and protein level, were shown to be reduced in *M. tuberculosis*–infected Tpl2−/− macrophages and Ifnar1−/− macrophages as compared with WT controls, and lowest levels of IL-10 mRNA and protein were detected in Tpl2−/− Ifnar1−/− *M. tuberculosis*–infected macrophages (Supplemental Fig. 3).

Elevated IFN-β mRNA expression and protein levels in *M. tuberculosis*–infected macrophages following TPL-2 deficiency were also examined in a recently developed macrophage cell line, the RAW 264.7 cell line (Fig. 4). RAW 264.7 cells were infected with *M. tuberculosis*, and the transcriptional and protein levels of IFN-β were analyzed. The results showed that IFN-β levels increased in RAW 264.7 cells following TPL-2 deficiency, with IFN-β mRNA expression and protein levels being significantly higher in Tpl2−/− Ifnar1−/− cells compared with WT controls. These findings suggest that the TPL-2–ERK pathway regulates the expression of IFN-β in *M. tuberculosis*–infected RAW 264.7 cells, and that this regulation is dependent on IFNAR signaling.
tuberculosis–infected Tpl2−/− macrophages were dependent on IFNAR expression (Supplemental Fig. 3B, 3C). This experiment demonstrates that a large component of the transcriptional changes observed in Tpl2−/− macrophages results from the increased levels of type I IFN signaling, affecting immunologically important genes such as Il12a, Ifna, and Ifngr1.

Type I IFN signaling contributes significantly to the increased susceptibility of Tpl2−/− mice to intracellular bacterial infection

Given the raised levels of type I IFN in L. monocytogenes–infected Tpl2−/− mice, and the IFNAR-dependent transcriptional perturbation of genes observed in Tpl2−/− macrophages, we hypothe-
sized that type I IFN overproduction in TPL-2–deficient animals may be contributing to their increased susceptibility to M. tuberculosis and L. monocytogenes infection in vivo. To test this hypothesis, we infected Tpl2−/−/Ifnar1−/− mice with M. tuberculosis or L. monocytogenes and compared their bacterial load with similarly infected single-knockout Tpl2−/− and Ifnar1−/− mice and WT control mice (Fig. 6). We chose the M. tuberculosis strains HN878 and BTB02-171 for infection as these strains are hyper-IFN inducing and would enhance any IFN-dependent differences between groups (36, 37) (J. Carmona and M. Saraiva, unpublished observations).

As previously seen, Tpl2−/− mice had higher bacterial loads compared with WT mice following infection with the different strains of M. tuberculosis or L. monocytogenes (Fig. 6A, 6B). Ifnar1−/− mice had similar lung bacterial levels to WT mice following infection with M. tuberculosis. Following L. monocytogenes infection, Ifnar1−/− mice had lower bacterial burdens in the spleen compared with WT mice (Fig. 6B), in line with previous reports (30–32). In all three infections, the bacterial loads in Tpl2−/−/Ifnar1−/− mice were significantly reduced compared with Tpl2−/− mice (Fig. 6A, 6B). Following M. tuberculosis infection, the increased bacterial burden in Tpl2−/− mice was completely abrogated in the absence of type I IFN signaling, with bacterial loads in the lungs of Tpl2−/−/Ifnar1−/− mice equivalent to those in WT and Ifnar1−/− control mice (Fig. 6A). L. monocytogenes–infected Tpl2−/−/Ifnar1−/− also had reduced bacterial loads compared with Tpl2−/− controls, to a level between the WT and Ifnar1−/− control groups (Fig. 6B). Measurement of IL-10 in the serum of the L. monocytogenes–infected mice revealed elevated concentrations of IL-10 in Tpl2−/− mice compared with WT controls (Fig. 6C), as shown earlier (Fig. 4C). This increase in IL-10 protein was abrogated in sera of Tpl2−/− mice crossed to Ifnar1−/− mice infected with L. monocytogenes (Fig. 6C). These levels reverted to that of WT and Ifnar1−/− mice, demonstrating that this elevation of IL-10 in Tpl2−/− mice sera was totally attributable to IFN-αβ. By contrast, IL-12p40 and IL-12p70 levels were significantly elevated in the serum of L. monocytogenes–infected Tpl2−/−/Ifnar1−/− mice, compared with WT, Tpl2−/−, and Ifnar1−/− controls (Fig. 6C), indicating an important role for type I IFN signaling in inhibiting IL-12 production, even in the absence of TPL-2–ERK1/2 signaling. Similar results were also seen in vitro L. monocytogenes–infected macrophages (Supplemental Fig. 4).

These data demonstrate a significant contribution of type I IFN signaling to the increased susceptibility of Tpl2−/− mice to intracellular bacterial infections.

**Discussion**

The macrophage plays a central role in the host response to intracellular bacterial infection. How macrophages perceive infection varies depending on the specific array of PRR engaged by a par-

**FIGURE 5.** Type I IFN signaling regulates transcription in Tpl2−/− macrophages. (A and B) Macrophages derived from WT, Ifnar1−/−, Tpl2−/−, and Tpl2−/−Ifnar1−/− mice were infected with M. tuberculosis H37Rv. At indicated time points, RNA was harvested, processed, and analyzed by microarray. Expression levels for the gene lists originally generated by comparison of M. tuberculosis–infected WT and Tpl2−/− macrophages and detailed in Fig. 3 were analyzed across the four strains and are shown here. See also Supplemental Fig. 2.
FIGURE 6. Type I IFN signaling contributes significantly to the increased susceptibility of Tpl2−/− mice to intracellular bacterial infection. (A) WT, Tpl2−/−, Ifnar1−/−, and Tpl2−/−Ifnar1−/− mice were infected with M. tuberculosis HN878 or M. tuberculosis BTB02-171 via the aerosol route. At the indicated times postinfection, lungs were harvested and bacterial loads were enumerated via serial dilution and plating. Graphs show one representative experiment of at least two for each M. tuberculosis strain, with at least three mice per group. Significance was determined using one-way ANOVA with Tukey post hoc test. (B) WT, Tpl2−/−, Ifnar1−/−, and Tpl2−/−Ifnar1−/− mice were infected with L. monocytogenes via the i.v. route. At the indicated time postinfection, spleens were harvested and bacterial loads were enumerated via serial dilution and plating. Graph shows one representative experiment of three, with at least three mice per group. Significance was determined using one-way ANOVA with Tukey post hoc test. (C) Serum from L. monocytogenes–infected WT, Tpl2−/−, Ifnar1−/−, and Tpl2−/−Ifnar1−/− mice was harvested at day 2 postinfection, and cytokines were measured by ELISA or bead array. Graphs show one representative experiment of at least two, with at least four mice per group. Significance was determined using one-way ANOVA with Tukey post hoc test. *p < 0.05, **p < 0.001.

Particular pathogen and on the signaling pathways activated downstream of these (49). We show in this study that, following infection of macrophages with M. tuberculosis, a set of gene transcripts is rapidly expressed, many of which have previously been described as targets of the TPL-2–ERK1/2 MAPK pathway (62–64). We now show that the TPL-2–ERK1/2 signaling pathway is important in mediating host resistance to M. tuberculosis and L. monocytogenes through negative regulation of type I IFN production.

Importantly, many of the ERK-inducible genes expressed, such as Fos, Egr1, and Imjd3, are transcription factors or transcriptional regulators implicated in regulation of cytokines in immune cells (50, 51, 54, 69, 70). Indeed, following TLR stimulation of macrophages, expression of the transcription factor FOS, downstream of TPL-2–ERK1/2 signaling, has been shown to regulate production of several cytokines, including IL-12, IL-10, and type I IFN (50, 51, 54). Dusp1 and Ier3, known regulators of signaling pathways including MAPK pathways, were also activated (71, 72). This profile suggested that the TPL-2–ERK1/2 pathway downstream of PRR may play an important role in the response to M. tuberculosis. We further investigated this by looking at the later transcriptional response in M. tuberculosis–infected macrophages. Several type I IFN transcripts were upregulated in Tpl2−/− cells, as was Il12a. Despite this increase in type I IFN transcripts in Tpl2−/− cells, many classically type I IFN-inducible transcripts (e.g., Mx1) were not upregulated in Tpl2−/− cells compared with WT sufficiently to pass the microarray filtering. This probably reflects the early time point (6 h) at which the analysis was done, as relatively little type I IFN feedback will have occurred by 6 h. Expression of Il10, Ifnb1, and Il1b was transcriptionally different in Tpl2−/− macrophages, as measured by qRT-PCR, despite not being sufficiently different to pass the microarray statistical filtering. Tnf mRNA levels were not affected by TPL-2 deficiency. However, it is likely that TNF-α and IL-1β and possibly IL-10 are also regulated at the posttranscriptional level. Interestingly, TPL-2 also appeared to regulate mRNA levels of Ifngr1 (which were reduced in Tpl2−/− macrophages), encoding a subunit of the IFN-γ receptor. Together, these findings further suggested that TPL-2–ERK1/2 signaling had important regulatory functions in the macrophage response to M. tuberculosis.

At the protein level, TPL-2–ERK1/2 was a positive regulator of TNF-α, IL-10, and IL-1β following M. tuberculosis infection of macrophages. In contrast, this pathway negatively regulated IL-12 and IFN-β. Importantly, this finding was not limited to bone marrow–derived macrophages, as ex vivo monocytes displayed the same phenotype. These findings are similar to results from previous reports that used purified TLR ligands to stimulate Tpl2−/− macrophages (52, 54–57, 59), with the exception of one report, in which TPL-2 was a positive regulator of IFN-β (58). However, type I IFN protein levels were only investigated at very early time points in that study (58), which might account for some of the difference with our results. Furthermore, that report examined mice lacking TPL-2 through an ENU-induced point mut-
tation (58), a process that may induce only partial reductions in signaling pathways.

The net effects of dysregulated cytokine production in mice lacking the TPL-2–ERK1/2 pathway resulted in increased susceptibility to intracellular bacterial infection, as seen by increased bacterial loads. We observed this finding following L. monocytogenes infection of mice, in agreement with a recent study (55). We now report the novel findings that loss of the TPL-2–ERK1/2 pathway resulted in exacerbated infection with M. tuberculosis, in which multiple strains with varying levels of virulence all showed similar phenotypes. The observation that Tpl2−/− mice on a Rag1−/− background are also more susceptible to M. tuberculosis and L. monocytogenes infection suggests that a defect in innate immunity is important for increased susceptibility. Interestingly, this provides an additional mechanism for increased pathogen load in infection of Tpl2−/− mice to that of a previously reported model of Toxoplasma gondii infection, in which T cell–intrinsic defects in the response to IL-12, IL-18, and TCR stimuli, leading to reduced IFN-γ production, in Tpl2−/− mice were suggested to be solely responsible for impaired host immunity (73). In our system, we did not see differences in IFN-γ protein levels in the serum of infected mice or by mRNA in lung (data not shown). It remains possible that any defect in CD4+ T cell IFN-γ production is obscured by IFN-γ produced by other sources that are not TPL-2–ERK1/2 regulated. Alternatively, a recent study found that CD4+ T cells that were unable to produce IFN-γ were still protective during tuberculosis (74). Our findings, although not wholly excluding a role for T cell defects in exacerbated M. tuberculosis and L. monocytogenes infection, support the idea that innate defects in immunity are important for lack of bacterial control in Tpl2−/− animals.

Because of the complex regulation of cytokine production in innate cells by the TPL-2–ERK1/2 pathway, the perturbation to the immune response in Tpl2−/− mice that leads to increased bacterial load in vivo could be attributed to a number of sources. Likely candidates to explain the heightened disease seen in Tpl2−/− mice infected with M. tuberculosis or L. monocytogenes were reduced levels of the host-protective cytokines TNF and IL-1β, or increased levels of type I IFN, which most likely play a detrimental role. We found evidence of increased levels of type I IFN in Tpl2−/− mice and macrophages following infection. Further dissection of the response of M. tuberculosis–infected macrophages, using Tpl2−/−/Ifnar1−/− cells, revealed that many of the changes seen in Tpl2−/− macrophages, both at the transcriptional and protein levels, were controlled by type I IFN signaling. Of note, this group included transcription of Ifng, suggesting that type I IFN may be at least partially responsible for downregulation of the IFN-γR during M. tuberculosis infection. This may in turn reduce macrophage activation in response to IFN-γ. In support of this, type I IFN has been shown to suppress macrophage activation through downregulating expression of the IFN-γR during L. monocytogenes infection (34, 75, 76). Altogether these results suggest that type I IFN signaling played a significant role in the dysfunctional response seen in the absence of TPL-2.

This led us to test the involvement of type I IFN signaling in the increased susceptibility of Tpl2−/− mice to bacterial infection. Upon infection with either M. tuberculosis or L. monocytogenes, we found that abrogation of type I IFN signaling restored protection of Tpl2−/− mice to WT or near WT levels. These results strongly point to the loss of negative regulation of type I IFN production as primarily responsible for the increased susceptibility of Tpl2−/− mice. Increased levels of type I IFN were not solely responsible for exacerbated bacterial loads in Tpl2−/− mice in all cases, however, as bacterial loads in Tpl2−/−/Ifnar1−/− mice were elevated slightly above Ifnar1−/− mice in some infections. A previous report has suggested that loss of IL-1β production, but not TNF-α, is responsible for increased bacterial loads in Tpl2−/− mice following L. monocytogenes infection (55). IL-1 is known to be important in host protection from L. monocytogenes (23, 24) and M. tuberculosis (19–22). However, it was not directly shown whether levels of IL-1β were reduced following in vivo L. monocytogenes infection of Tpl2−/− mice, nor whether the loss of IL-1β seen in vitro was a direct effect of TPL-2 deficiency or was mediated through another factor. We saw little difference in the transcriptional levels of TNF-α in the lung (data not shown) or protein levels in serum. However, we also saw little difference in IL-1β. Nonetheless, this does not exclude a role for reduced levels of IL-1β contributing to exacerbated disease. Our results suggest, however, that reduced IL-1β in Tpl2−/− mice is likely to be an indirect effect of TPL-2 deficiency mediated by increased amounts of type I IFN. Indeed, we find, and others have also reported, that type I IFN is a potent suppressor of IL-1 production, in a partially IL-10–dependent manner (Supplemental Fig. 2 and data not shown) (20, 66).

Unexpectedly, levels of IL-10 were increased in serum of infected Tpl2−/− animals, in contrast to results seen with macrophages in vitro. This increase in IL-10 was dependent on type I IFN signaling, as infected Tpl2−/−/Ifnar1−/− mice did not have increased serum levels of IL-10. This suggests that increased levels of type I IFN are capable of overcoming the requirement for TPL-2–ERK1/2 signaling for IL-10 production in vivo. Further supporting this idea, L. monocytogenes– or M. tuberculosis–infected Tpl2−/− macrophages in vitro produced increased levels of IL-10 in response to exogenous IFN-α or IFN-β, indicating that these cells are still responsive to type I IFN, although IL-10 production was still impaired compared with WT macrophages. In addition, it is possible that increased type I IFN produced by myeloid cells in vivo induces IL-10 production from other cell types that do not require TPL-2 for IL-10 induction, but that are responsive to type I IFN, or that very high levels of type I IFN can overcome the TPL-2–ERK1/2 dependency of IL-10 production. Altogether, these results suggest that, in vivo, promotion of IL-10 production is at least one mechanism by which the excess levels of type I IFN seen in TPL-2–deficient mice could lead to immunosuppression and hence increased susceptibility to M. tuberculosis and L. monocytogenes infection (20, 66–68).

In summary, we show that the TPL-2–ERK1/2 signaling pathway in innate immune cells plays an important role in controlling intracellular bacterial infections caused by M. tuberculosis and L. monocytogenes. Despite the role of this pathway in regulating production of multiple cytokines, it is its ability to negatively regulate the production of type I IFN that is crucial in promoting host resistance to these bacterial pathogens. Because of the role of the TPL-2–ERK1/2 pathway in regulating TNF-α production, it has attracted attention as a possible therapeutic target for treating inflammatory conditions such as rheumatoid arthritis (77). Our results suggest that great care will be necessary in targeting this pathway as it may lead to unwanted exacerbation of, or increased susceptibility to, certain bacterial infections.

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References


