ORIGINAL RESEARCH

TBK1 Is Required for Host Defense Functions Distinct from Type I IFN Expression and Myeloid Cell Recruitment in Murine *Streptococcus pneumoniae* Pneumonia

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Abstract

Bacterial pneumonia induces the rapid recruitment and activation of neutrophils and macrophages into the lung, and these cells contribute to bacterial clearance and other defense functions. TBK1 (TANK-binding kinase 1) performs many functions, including activation of the type I IFN pathway and regulation of autophagy and mitophagy, but its contribution to antibacterial defenses in the lung is unclear. We previously showed that lung neutrophils upregulate mRNAs for TBK1 and its accessory proteins during *Streptococcus pneumoniae* pneumonia, despite low or absent expression of type I IFN in these cells. We hypothesized that TBK1 performs key antibacterial functions in pneumonia apart from type I IFN expression. Using TBK1 null mice, we show that TBK1 contributes to antibacterial defenses and promotes bacterial clearance and survival. TBK1 null mice express lower concentrations of many cytokines in the infected lung. Conditional deletion of TBK1 with LysMCre results in TBK1 deletion from macrophages but not neutrophils. LysMCre TBK1 mice have no defect in cytokine expression, implicating a nonmacrophage cell type as a key TBK1-dependent cell. TBK1 null neutrophils have no defect in recruitment to the infected lung but show impaired activation of p65/NF-κB and STAT1 and lower expression of reactive oxygen species, IFNγ, and IL12p40. TLR1/2 and 4 agonists each induce phosphorylation of TBK1 in neutrophils. Surprisingly, neutrophil TBK1 activation *in vivo* does not require the adaptor STING. Thus, TBK1 is a critical component of STING-independent antibacterial responses in the lung, and TBK1 is necessary for multiple neutrophil functions.

Keywords: pneumonia; neutrophil; interferon; cytokine; kinase

Bacterial pneumonia induces recruitment of many cell types and elaboration of mediators including type I and II IFNs, reactive oxygen species (ROS), and inflammatory cytokines. Neutrophils are critical early responders to bacterial pneumonia, and they clear pathogens both directly by phagocytosis and indirectly by activation of other cell types. *Streptococcus pneumoniae* in the mouse or human lung rapidly induces brisk neutrophil recruitment and activation. Upon recruitment, activated neutrophils use multiple signaling pathways to regulate tasks such as migration, phagocytosis, ROS production, and transcription of mRNA. In particular, signaling kinases, including the MAP kinase family, JNK, and mTOR, drive critical cell processes including translation, mitochondrial activity, and cytoskeletal rearrangement (1). Neutrophils are an attractive target for host-directed therapeutics in settings where they cause significant tissue injury. Thus, understanding their signaling is useful for developing neutrophil-directed drugs that tune their responses.

Type I and II IFNs play overlapping and important roles in bacterial pneumonia, and their expression is tightly regulated. Type I IFN (IFNβ and the many isoforms of IFNα)

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can be induced by bacterial and viral components as well as host damage-associated molecular patterns (DAMPs); type I IFNs induce receptive cells to restrict viral replication as well as potentiate the cytotoxic activity of natural killer (NK) and T cells (2). Type I IFN expression is controlled by the cooperative action of transcription factors from the IRF (interferon regulatory factor) and NF-κB families. Viral and bacterial ligands are sensed by PRRs (pathogen recognition receptors) such as the TLR family, RIG-I/MDA-5, or cGAS/STING, leading to activation of the kinase TBK1 (TANK-binding kinase 1). TBK1 phosphorylates IRF3 and, in some cells, IRF7, causing them to translocate to the nucleus and drive transcription of the IFNα and IFNβ loci. The promoters for IFNα/β also have binding sites for NF-κB p65/RelA, and under some circumstances TBK1 may directly phosphorylate and activate p65 (3). However, when studied by conditional deletion in individual cell types, TBK1 performs distinct, type I IFN-independent functions in T cells, B cells, adipocytes, and dendritic cells (4–7), demonstrating that TBK1 contributes to other pathways.

Type II IFN (IFNγ) potently activates macrophages, polarizing them toward antibacterial responses and promoting phagocytosis and bactericidal functions. IFNγ is commonly expressed by T cells and NK cells downstream of the transcription factor Tbx21/T-bet. Neutrophils can express IFNγ in the setting of bacterial pneumonia (including both *Streptococcus* and *Staphylococcus*), but T-bet expression in neutrophils has not been observed, suggesting that another pathway generates IFNγ in this context (8–10).

In examining the transcriptome of purified neutrophils from *S. pneumoniae*–infected mouse lungs, we made the paradoxical observation that neutrophils upregulate mRNA expression of multiple components of the TBK1 pathway but do not appear to express significant amounts of type I IFN (8). Specifically, activated neutrophils upregulated the mRNA of TBK1 itself, the related kinase IKKε, upstream activators (DAI and RIG-I), TBK1 scaffolds (TANK, NEMO), and TBK1 phosphorylation targets (IRF7). But although the lung neutrophils expressed high concentrations of multiple cytokine mRNAs, the mRNAs for IFNβ or the many IFNα isoforms were expressed at low concentrations and not significantly upregulated during *S. pneumoniae* infection, although IFNγ mRNA and protein were significantly produced. The transcriptional state of these neutrophils was more consistent with an IFN type I–stimulated phenotype than an IFN type I–producing phenotype. These findings are in agreement with a study by Ericson and colleagues examining neutrophil activation in sterile inflammation, such as thioglycolate- or uric acid–induced peritonitis (11). Taken together, these observations suggest that activated neutrophils upregulate the TBK1 pathway to execute distinct functions unrelated to viral control or IFN I production.

In this study, we test the hypothesis that TBK1, a known critical component of antiviral responses, mediates multiple aspects of the myeloid response to *S. pneumoniae* during pneumonia. Our data show that TBK1 is required for host defense functions apart from type I IFN expression or cell recruitment, including ROS production, bacterial clearance, transcription factor activation, and elaboration of multiple cytokines. TBK1 deletion produces a different phenotype than deletion of either IFNAR (type I IFN receptor) or STING (12, 13). We believe that these are the first studies to examine the function of TBK1 in neutrophils during inflammation and an innate immune response. These findings highlight the role of TBK1 in host defense against bacteria in the lung.

Methods

Mice

Sv129 $TBK1^{+/\Delta}$ mice were the kind gift of Dr. Perry Hall (Pfizer) and were crossed to generate Sv129 wild type (WT) and $TBK1^{\Delta/\Delta}$, also referred to as TBK1 knockout (KO). C57Bl/6 TBK1lox/lox mice were the kind gift of Dr. Katherine Fitzgerald (University of Massachusetts) and were crossed with LysM-Cre mice from Jackson Laboratories (stock 004781), described previously (14); these mice are heterozygous for LysM-Cre. STING KO mice were from Jackson Laboratories and were the kind gift of Dr. Uma Nagarajan. $gp91^{phox}$ KO mice are maintained in our colony and have been described previously (10). Colonies of all genotypes were maintained at the University of North Carolina at Chapel Hill. All mouse lines were bred and housed in ventilated cages in pathogen-free facilities. Experiments involving animals were conducted in accordance with recommendations from the American Association for Laboratory Animal Science. Procedures were conducted using protocols approved by the University of North Carolina School of Medicine Institutional Animal Care and Use Committee.

Bacterial Infections

Eight- to 12-week-old mice were infected by intratracheal administration into the left lung of *S. pneumoniae* (serotype 19, ATCC 49619) suspended in PBS, optical density (OD) 0.9, 2.3 μl/gm body weight as described previously (8). The range of colony-forming units was $1.2–3.3 \times 10^7$ per mouse. For *in vitro* infection, 10^6 bone marrow cells at a density of 10^5/ml were mixed with *S. pneumoniae* OD 0.8 at a final dilution of 1:20 and incubated at 37°C for 4 hours in the presence of brefeldin A before permeabilization, staining, and fixation.

BAL Fluid Collection and Analysis

The lungs of killed mice were lavaged once with 1 ml PBS/2 mM EDTA for cytokine analysis or 5×1 ml for cell analysis. Cytokines were measured by Bio-Plex Pro Mouse Cytokine Group 1 Panel 23-Plex (Bio-Rad). Mouse IFNβ and IFNα ELISAs were from InvivoGen. BAL cell differentials and protein concentration were measured as described (10). For purification of BAL neutrophils, BAL cells were labeled with Ly6G-FITC or Ly6G-biotin and purified with anti-FITC or antibiotin beads (Miltenyi).

Flow Cytometry

Lungs were digested to single-cell suspensions by intratracheal instillation of 5 mg/ml collagenase I (Worthington Biochemical Corporation) and 0.25 mg/ml DNase I (Sigma) at 37°C for 30 minutes, followed by mechanical disruption, as previously described (15). Details of antibody and ROS staining are in the online supplement.

Immunoblotting and Antibodies

Cells were lysed in complete RIPA buffer, resolved by SDS-PAGE, and blotted as described previously (14). For phospho-specific antibodies, membranes were blocked with 1% BSA in TBST before probing. Antibodies are listed in the online supplement.

Statistical Analysis and Graphics

Error bars represent the SEM. For qRT-PCR, all samples were run in technical (assay)

triplicate or duplicate. All figures depict experiments that have been replicated a minimum of two times. For Kaplan-Meier survival curves, significance was computed by Gehan-Breslow-Wilcoxon test. ANOVA and two-tailed unpaired *t* test were performed in GraphPad Prism. Asterisks indicate significant differences between genotypes or treatments, and the level of significance is indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, and $^{****}P < 0.001$.

Results

TBK1 Deletion Impairs the Host Response to *S. pneumoniae*

Based on our observation that TBK1 and TBK1 pathway gene mRNAs are upregulated in neutrophils during pneumonia (8), we examined the requirement for TBK1 in a setting in which neutrophils contribute significantly to pathogen control. Sv129 WT and TBK1 KO mice (also known as $TBK1^{\Delta/\Delta}$) (16) were challenged with intratracheal inoculation of *S. pneumoniae*. Relative to WT mice, TBK1 KO mice had worse survival (Figure 1A) but no observable difference in post-infection weight loss or recovery of weight in those who survived (Figure 1B). At 24 hours post inoculation (p.i.), TBK1 KO mice displayed impaired clearance of bacteria from the lung (Figure 1C). By 48 and 96 hours p.i., bacterial clearance was nearly complete and similar between the two genotypes (Figure 1C).

To determine if macrophages are the important site of TBK1 expression in clearance, LysMCre/TBK1flox mice (termed M-TBK1 KO) were compared with Cre(−)/TBK1flox mice (termed littermate WT mice). Macrophages of M-TBK1 KO mice do not express TBK1, whereas neutrophil TBK1 expression appears intact in these mice (*see* Figure E1 in the online supplement) (14). Interestingly, the defect in clearance at 24 hours is not observed in M-TBK1 KO mice compared with littermate WT mice (Figure 1D).

No significant differences between WT and KO mice were observed in BAL fluid protein concentrations (Figure 1E) at 24 hours or total BAL cells (Figure 1F) at 24 or 48 hours p.i. Examination of BAL cell populations using cytospins showed that BAL fluid from TBK1 KO mice contained a smaller percentage and number of macrophages in BAL at 24 hours but no significant difference in the number of BAL neutrophils (Figures 1G and 1H); by 48 hours p.i., differences between WT and KO mice were no longer significant. Single-cell digests of the lung revealed a similar difference in the percentage and number of lung macrophages (defined as $CD45^{+}/CD64^{+}/Ly6G^{-}$ cells) between genotypes and no difference in $Ly6G^{+}/CD11b^{+}$ lung neutrophils (Figures 1I and 1J). Taken together, these data suggest that TBK1 contributes to bacterial clearance, survival, and macrophage kinetics but not to neutrophil recruitment. Furthermore, the defect in clearance is not due to TBK1 expression in macrophages.

TBK1 Is Required for Expression of Multiple Cytokines in the Infected Alveolar Space

Because TBK1 is required for type I IFN expression in many contexts, we examined the requirement for TBK1 in alveolar cytokine expression. TBK1 KO mice expressed lower concentrations of IFNγ, GM-CSF, G-CSF, KC, MCP1, IL12p70, IL1α, IL1β, RANTES, and IL6 protein at 24 hours p.i. relative to WT mice (Figure 2A); these differences between genotypes were no longer apparent at 48 hours p.i., when these mediators are decreasing. Expression of TNFα and the chemokines MIP-1α and MIP-1β was not different between WT and TBK1 KO (Figure 2A and Table E1) or the cytokines IL2, IL17A, and IL10 (Table E1). Mice that did not undergo instillation or that received PBS instillation had BAL cytokine concentrations below the assay range for nearly all the cytokines in this 23-plex panel, indicating induction in the setting of *S. pneumoniae* infection (Table E1).

Because multiple lung macrophage populations and recruited monocytes contribute to lung cytokine expression, we examined BAL cytokine concentrations in LysMCre/TBKflox mice, in which TBK1 is efficiently deleted in macrophages but not in neutrophils (Figure E1 and Reference 14). No difference in BAL cytokine expression was observed between LysMCre/TBK1flox mice and littermate Cre(−)/TBK1flox mice at 24 hours p.i. (Figure 2B). These data suggest that at this time point, macrophage TBK1 is not required for the bulk of BAL cytokine expression. Taken together, these data suggest that TBK1 in nonmacrophage cells contributes significantly to the elaboration of cytokines in the alveolar space in the early response to *S. pneumoniae*.

Measurement of type I IFN expression in the BAL showed that IFNβ protein was not increased in the alveolar space of *S. pneumoniae*–infected mice relative to PBS-treated mice (Figure 2C), and we could detect no difference in TBK1 KO or LysMCre/TBK1flox mice compared with control mice. mRNA concentrations of IFNα, IFNβ, and the coregulated chemokine CXCL10 in whole-lung homogenates were induced by *S. pneumoniae*, but TBK1 KO mice showed only nonsignificant trends toward lower expression of type I IFN and CXCL10 24 hours after infection (Figure 2D). At 12 hours after infection, there is actually more IFNα4 and CXCL10 mRNA in the KO lung compared with the WT. By 48 hours, this difference is gone, and expression of IFNα4 and IFNβ1 mRNAs has returned to preinfection concentrations (Figure 2E).

TBK1 Is Activated in Neutrophils by Bacterial Ligands and Bacteria

Because of the defect in many neutrophil-associated cytokines in TBK1 KO mice (Figure 2A), we examined TBK1 activation in neutrophils. TBK1 kinase activity correlates closely with phosphorylation of serine 172 (S172) in the kinase domain (17). When BAL neutrophils of *S. pneumoniae*–infected mice were purified with anti-Ly6G beads at 24 hours p.i., TBK1 S172 phosphorylation was clearly observed by immunoblot (Figure 3A). We next asked whether purified TLR ligands representative of gram-negative or gram-positive bacteria could activate neutrophil TBK1 *in vitro*. We observed TBK1 S172 phosphorylation in bead-purified neutrophils stimulated *in vitro* with either the TLR4 ligand LPS or TLR2 ligand PAM3Cys (Figure 3B).

The STING pathway activates TBK1 in response to cytosolic DNA or cyclic dinucleotides produced by bacteria or the host enzyme cGAS (reviewed in Reference 18). To compare TBK1 phosphorylation upon activation of TLR or STING pathways, we stimulated mouse bone marrow cells *in vitro* with the TLR2 ligand PAM3Cys or the STING activator DMXAA and assessed TBK1 phosphorylation by flow cytometry. We used TBK1 KO mice as a negative control for both background fluorescence and cross-reactivity with S172 on the related kinase IKKε (IKK epsilon). Relative to mice lacking TBK1, we observed measurable phospho-S172 TBK1 in unstimulated bone marrow neutrophils ($Ly6G^{++}/Ly6C^{-}$ cells)

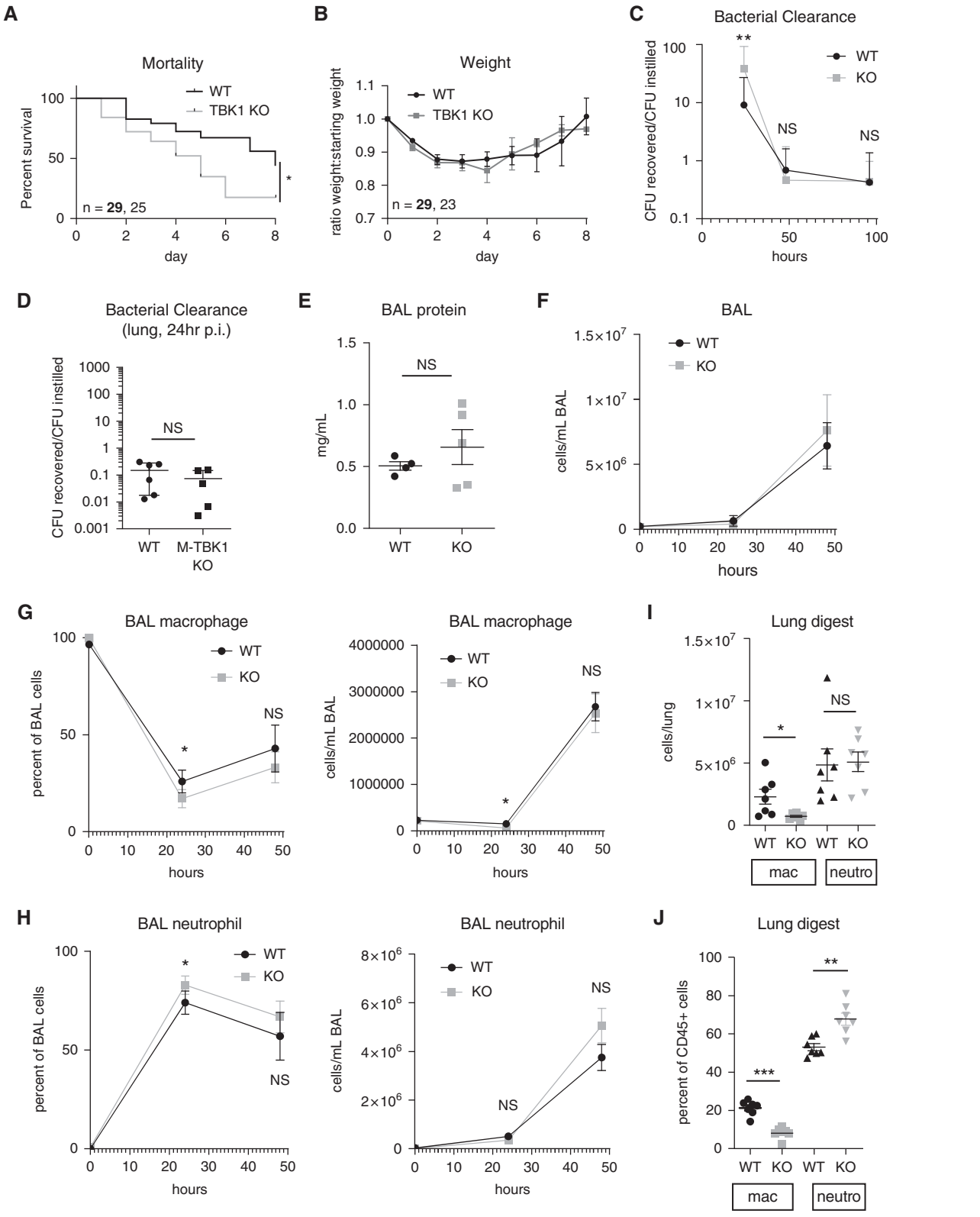

Figure 1. TBK1 (TANK-binding kinase 1) deletion decreases survival and impairs bacterial clearance in *Streptococcus pneumoniae* pneumonia. (*A*) Survival and (*B*) body weight curve for wild-type (WT) or TBK1 knockout (KO) mice infected intratracheally with *S. pneumoniae.* (*C*) Bacterial clearance was measured at 24, 48, and 96 hours post inoculation (p.i.) from lung homogenates of WT or TBK1 KO mice or (*D*) of WT and M-TBK1 KO mice. (*E*) Measurements of BAL fluid protein concentration and (*F*) BAL cell counts measured at 0, 24, and 48 hours p.i.

and monocytes ($Ly6G^-/Ly6C^+$) (Figure 3C). Stimulation with PAM3Cys caused marked upregulation of pTBK1 signal in neutrophils and monocytes (Figure 3C; gating strategy shown in Figure E2). The STING ligand DMXAA only modestly activated TBK1 phosphorylation in bone marrow monocytes *in vitro* and did not significantly increase TBK1 phosphorylation in neutrophils. When WT bone marrow was stimulated with live *S. pneumoniae in vitro* or PAM3Cys, we observed no induction of TBK1 phosphorylation by *S. pneumoniae* in neutrophils and only modest phosphorylation in monocytes compared with unstimulated and PAM3Cys treatment (Figure 3D).

We examined the necessity for STING in activation of TBK1 *in vivo* using STING KO mice, because STING is believed to be required for TBK1/IRF3 activation in *S. pneumoniae* pneumonia (19). The percentage and number of neutrophils in the lung digest were lower in the STING KO mice relative to WT C57Bl/6 mice (Figures 3E and 3F). Surprisingly, lung macrophages and neutrophils from infected STING KO mice showed elevated phosphorylation of TBK1 S172 at 24 hours p.i., suggesting that the STING pathway may repress rather than promote TBK1 activation in this specific setting (Figure 3G). Taken together, these data suggest that bacterial ligands and bacterial infection lead to the activating phosphorylation of TBK1 in neutrophils and that STING may contribute to the regulation of this phosphorylation in unexpected ways.

TBK1 Is Required for Neutrophil IFNγ and ROS Production

Because multiple BAL cytokines are decreased in TBK1 KO mice despite unaltered neutrophil recruitment, we examined the contribution of TBK1 to neutrophil functions relevant to cytokine expression. We used intracellular cytokine staining and flow cytometry to assess IFNγ expression in neutrophils and macrophages. At 24 hours p.i., only a very small percentage of lung macrophages had observable IFNγ expression (<2%), and no difference was seen between TBK1 KO and WT mice (data not shown). In contrast, 20–40% of WT neutrophils expressed IFNγ protein. Importantly, TBK1 KO neutrophils had impaired expression of IFNγ after *S. pneumoniae* infection, as evidenced by both a lower percentage of IFNγ+ cells and a lower degree of expression (measured by median fluorescence intensity) (Figure 4A). TBK1 was also required for expression of IL12p40 in neutrophils (Figure 4A). Of note, this expression of IFNγ seems to require multiple or tissue-derived signals, because bone marrow neutrophils exposed to live *S. pneumoniae* in tissue culture did not require TBK1 for IFNγ expression (Figure 4B). When we examine M-TBK1 KO mice and WT littermates, there was no difference in the percentage of IFNγ-expressing neutrophils in the lung and only a nonsignificant trend toward decreased IFNγ median fluorescence intensity (Figure 4C).

Because the generation of ROS has been implicated in driving IFNγ expression (10) and in killing of bacteria, ROS production in lung neutrophils and macrophages 24 hours after *S. pneumoniae* infection was assessed using flow cytometry. $gp91^{phox}$ is a critical component of the nicotinamide adenine dinucleotide phosphate (NAPDH) oxidase complex and neutrophils and macrophages from mice deficient in $gp91^{phox}$ have markedly impaired ROS production and bacterial killing (20, 21). $Gp91^{phox}$-deficient mice served as a negative control for ROS production. TBK1 KO lung neutrophils had ~50% lower ROS intensity relative to WT mice at 24 hours p.i. (Figure 4D). Lung macrophages from TBK1 KO mice also showed a significant, albeit smaller, reduction in ROS intensity at this time point. When M-TBK1 KO mice are examined, there is no significant difference in lung neutrophil ROS expression, suggesting that macrophage TBK1 is not required for neutrophil ROS production in this context (Figure 4E). Similar to IFNγ expression, bone marrow neutrophils from TBK1 KO mice do not show a significant impairment in ROS production when stimulated with live *S. pneumoniae in vitro* (Figure 4F).

TBK1 Regulates Neutrophil Transcription Factor Expression and Activation

Transcriptomic studies have shown that activated neutrophils synthesize new mRNA and regulate gene expression in response to environmental and pathogenic stimuli (8, 11). Because transcription factors represent a point of regulation in this process, and because TBK1 directly regulates transcription factors such as IRF3, IRF7, and p65/RelA NF-κB (reviewed in Reference 3), we examined transcription factor activation in lung neutrophils after *S. pneumoniae* infection. Bead-purified lung neutrophils from the BAL of infected TBK1 KO mice had less phosphorylation of STAT1 pY701 relative to WT, as assessed by immunoblot (Figure 5A). Protein concentrations of IκBα were not perturbed in neutrophils of TBK1 KO mice (Figure 5A), suggesting that TBK1 does not regulate NF-κB via IκBα. When STAT1 Y701 and p65/RelA S536 phosphorylation of neutrophils from lung digests was measured by flow cytometry, TBK1 KO lung neutrophils showed lower phosphorylation of both transcription factors (Figure 5B), suggesting that TBK1 regulates activation of neutrophil NF-κB and STAT1. Although STAT1 Y701 is typically directly phosphorylated by Jak family tyrosine kinases, TBK1 has been shown to phosphorylate STAT1 at the nearby T749. We suggest that TBK1-induced phosphorylation at T749 indirectly regulates phosphorylation at Y701 (22), although we cannot test this hypothesis because there are no antibodies specifically recognizing STAT1 pT749.

Our prior transcriptomic analysis showed that *S. pneumoniae* infection causes lung neutrophils to upregulate mRNA for the IRF family members IRF7 and IRF5, whereas the IFNβ-driving transcription factor IRF3 is largely unaffected (8). IRF7 has defined functions in antiviral responses, whereas IRF5 is believed to drive inflammatory and antibacterial responses (23). Relative to WT lung neutrophils, we observe a small difference in concentrations of macrophage IRF7 but no difference in neutrophil IRF7 protein at 24 hours p.i. (Figure 5C). Surprisingly, we observe marked upregulation of IRF5 in TBK1 KO lung neutrophils at 24 hours p.i., suggesting that TBK1 is dispensable for the expression of IRF5 in this context and may actually inhibit pathways important in IRF5 expression or protein stability. These data show that TBK1 is required for

Figure 1. (*Continued*). (*G* and *H*) Neutrophils and macrophages were identified in BAL by cytospin and Diff-Quik staining. (*I*) The number and (*J*) percentage of $CD45^+$ leukocytes that were macrophages or neutrophils and (*J*) the number of lung macrophages and neutrophils per lung were identified in single-cell lung digests by flow cytometry; macrophages were defined as $CD45^+/CD64^+/Ly6G^-$; neutrophils were defined as $CD45^+/CD64^-/Ly6G^+/CD11b^+$. $n = 6–8$ mice per group in three replicates. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.005$. CFU = colony-forming unit; NS = not significant.

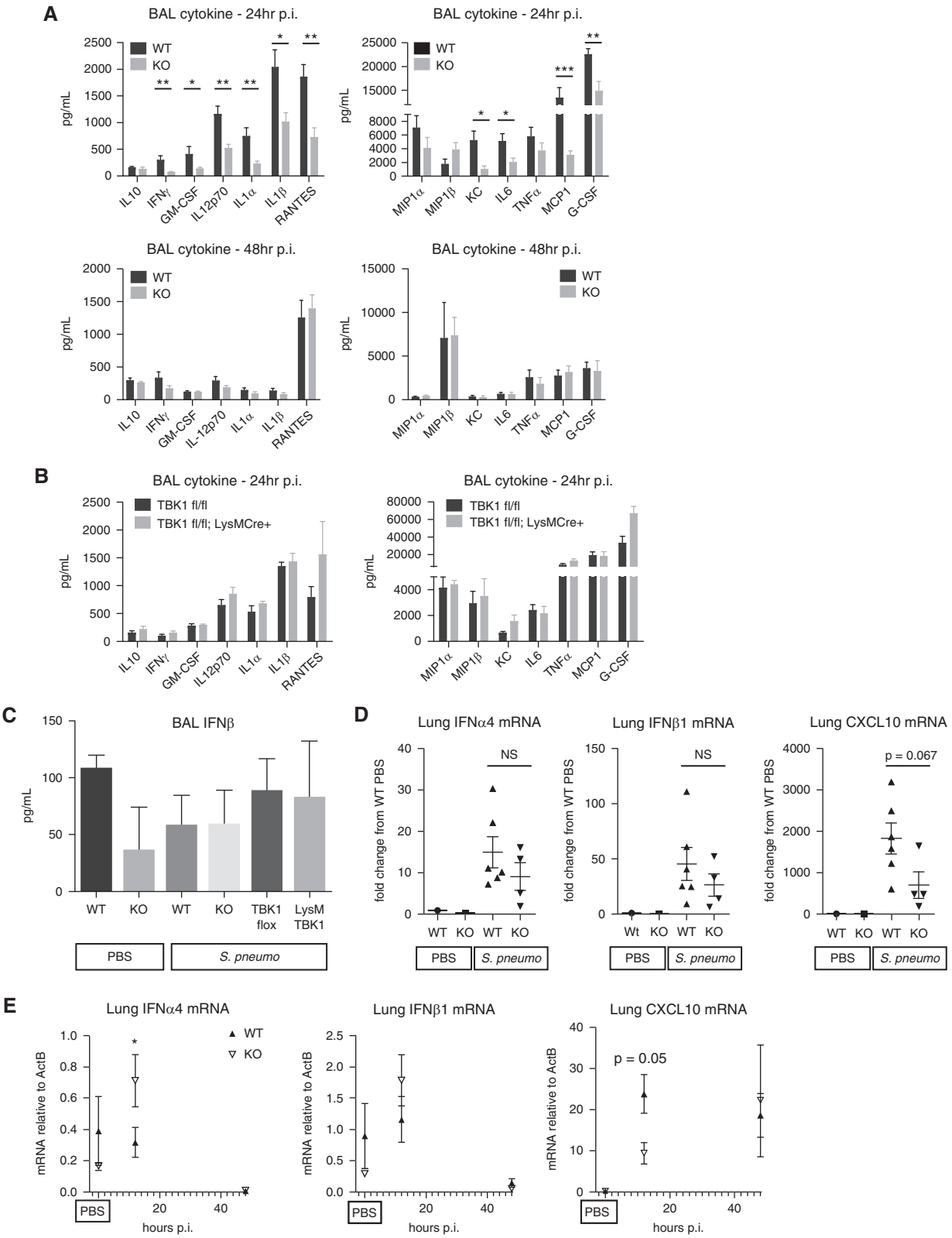

Figure 2. TBK1 is required for expression of multiple cytokines in the infected alveolar space. (*A*) BAL cytokines from *S. pneumoniae*–infected WT or TBK1 KO mice at 24 or 48 hours p.i were measured by ELISA. (*B*) BAL cytokines from Cre(−)/TBK1flox or LysMCre(+)/TBK1 flox mice at 24 hours p.i. were measured by ELISA as in *A*. (*C*) BAL IFNβ from PBS-treated or *S. pneumoniae*–infected WT, TBK1 KO, TBK1flox, or LysMCre(+)/TBK1flox mice were measured by ELISA. No significant differences between genotypes were observed. (*D*) qRT-PCR of indicated

regulation of expression and activation of multiple transcription factors in lung neutrophils during *S. pneumoniae* pneumonia.

Discussion

In responding to a pathogen, neutrophils and macrophages must sense multiple extracellular signals, including pathogen- and damage-associated molecular patterns (PAMPs and DAMPs), integrate these signals, and then execute a variety of antipathogen functions such as phagocytosis, ROS production, and cytokine secretion. These functions promote pathogen clearance as well as adaptive immunity. The signaling kinase TBK1 represents the convergence point of many PRR and DAMP receptors and thus is a critical node for the cell's decision to activate various arms of the host defense program. Studies in macrophages and epithelial cells have defined TBK1's contribution to type I IFN expression, particularly in response to viruses, TLR ligands, and cytoplasmic DNA. TBK1 also promotes mitophagy and in some cell types drives activation-induced glycolysis. In this study, we examine TBK1 function against the common respiratory pathogen *S. pneumoniae*. Our results show strikingly that TBK1 contributes significantly to bacterial clearance, inflammatory cytokine expression, and ROS production in the *S. pneumoniae*–infected lung. These functions appear unaffected when TBK1 is deleted from macrophages with LysM-Cre, suggesting that other cell types such as neutrophils or epithelial cells are using TBK1 to express inflammatory mediators. These functions also occur in a context where type I IFN protein is not significantly induced and is distinct from the phenotype of IFNAR KO mice, suggesting that TBK1 executes these functions independent of type I IFN.

Is TBK1 functioning primarily in neutrophils or primarily in another cell type, such as the respiratory epithelium? Our data support at least two nonexclusive models of how TBK1 promotes anti-*Streptococcus* defense (Figure 6). In one model, neutrophil-intrinsic TBK1 receives pathogen signals from PRRs such as TLR2 or STING and directly activates downstream transcription factors that drive cytokine and ROS production (Figures 3B and 6A). In an alternative, neutrophil-extrinsic, model, other cell types such as epithelial cells and alveolar macrophages require TBK1 to express cytokines that activate their cognate receptors on neutrophils, leading to activation of neutrophil JAK/STAT pathway and downstream transcription (Figure 6B). This model is supported by our observation that TBK1 is required for neutrophil IFNγ and ROS production *in vivo* (Figures 4A and 4D) but not when TBK1 neutrophils are stimulated *in vitro* with live bacteria (Figures 4B and 4F). These data suggest a role for TBK1-dependent signals from the lung environment to promote neutrophil function. In addition, the maturity of the neutrophils may contribute to the function of TBK1. These models are not mutually exclusive, and parsing them will require the development of mouse models or other methods that allow for transplantation or manipulation of TBK1-null neutrophil precursors.

Is there a contribution of macrophage TBK1 to the phenotype seen in these mice? We observe a defect in macrophage numbers in BAL and total lung at 24 hours p.i., which may reflect a defect in recruitment of monocyte-derived macrophages similar to the defect seen when TBK1 is deleted from macrophages before influenza infection (14). Lung macrophages also have a defect in ROS production (Figure 4D), which may also contribute to the defect in clearance of *S. pneumoniae* in the absence of TBK1.

Do TLRs and/or STING activate TBK1 in *S. pneumoniae* pneumonia? In bone marrow–derived macrophages exposed *in vitro* to live *S. pneumoniae*, TBK1 activation is driven by bacterial DNA that enters the macrophage cytosol and is sensed by DAI and STING (19). In contrast, STING is completely dispensable for TBK1 activation in both neutrophils and macrophages *in vivo*, suggesting that STING is not the primary activator (and may in fact be inhibitory to TBK1) in response to *S. pneumoniae*. Our observation (Figure 3D) that neutrophils and macrophages from STING-null mice have increased TBK1 phosphorylation during pneumonia may reflect loss of STING-dependent expression of negative regulators of the TLR/TBK1 pathway, as has been observed in other systems (24). TLR2 is activated by multiple bacterial ligands and can activate TBK1 *in vitro* (25); TLR2 KO mice infected with *S. pneumoniae* demonstrate no impairment in bacterial clearance and no increase in mortality, suggesting that TLR2 is not the sole activator of TBK1 in this context (26). *In vitro*, TLR2 ligands drive neutrophil TBK1 phosphorylation as potently as TLR4 ligands, whereas the STING ligand DMXAA only weakly promotes neutrophil TBK1 phosphorylation (Figure 3B), again arguing that pathways other than STING activate neutrophil TBK1 in this context.

Because neutrophil recruitment is not impaired in infected TBK1 KO mice, we examined activation of neutrophil transcription factors from the STAT, IRF, and NF-κB family. Neutrophils from TBK1 KO mice fail to activate STAT1 and p65 NF-κB but have largely intact concentrations of IRF7. Although TBK1's direct phosphorylation of IRF3 and IRF7 is well documented, TBK1 has been shown to phosphorylate other transcription factors, such as p65/RelA, cRel, STAT6, and STAT3, only under specific circumstances and in limited cell types (reviewed in Reference 3). Intriguingly, TLR2 stimulation of macrophages activates TBK1 but does not promote IRF3 phosphorylation (25), suggesting that TLR2 ligands direct TBK1 either to another transcription factor (such as RelA) or to a non-transcriptional output.

In examining cytokine expression by neutrophils, we found that TBK1 is required for neutrophils to produce IFNγ and IL12. The transcriptional regulation of IFNγ remains obscure, as multiple investigations have failed to show that neutrophils express measurable amounts of Tbx21/Tbet, the transcription factor that drives IFNγ in T cells and NK cells (8, 11). As discussed above, these results suggest that neutrophil TBK1 either activates an unknown transcription factor that drives IFNγ and IL12p40 mRNA expression or that it regulates their

Figure 2. (*Continued*). genes from lung homogenates at 24 hours p.i. (*E*) RT-PCR of indicated genes from lung homogenates at 12 and 48 hours p.i. or in PBS-treated control mice. $n = 4$–8 mice per infected group and $n = 2$–3 mice per PBS group, and data are indicative of at least three (*A*) or two (*B–E*) independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.005$.

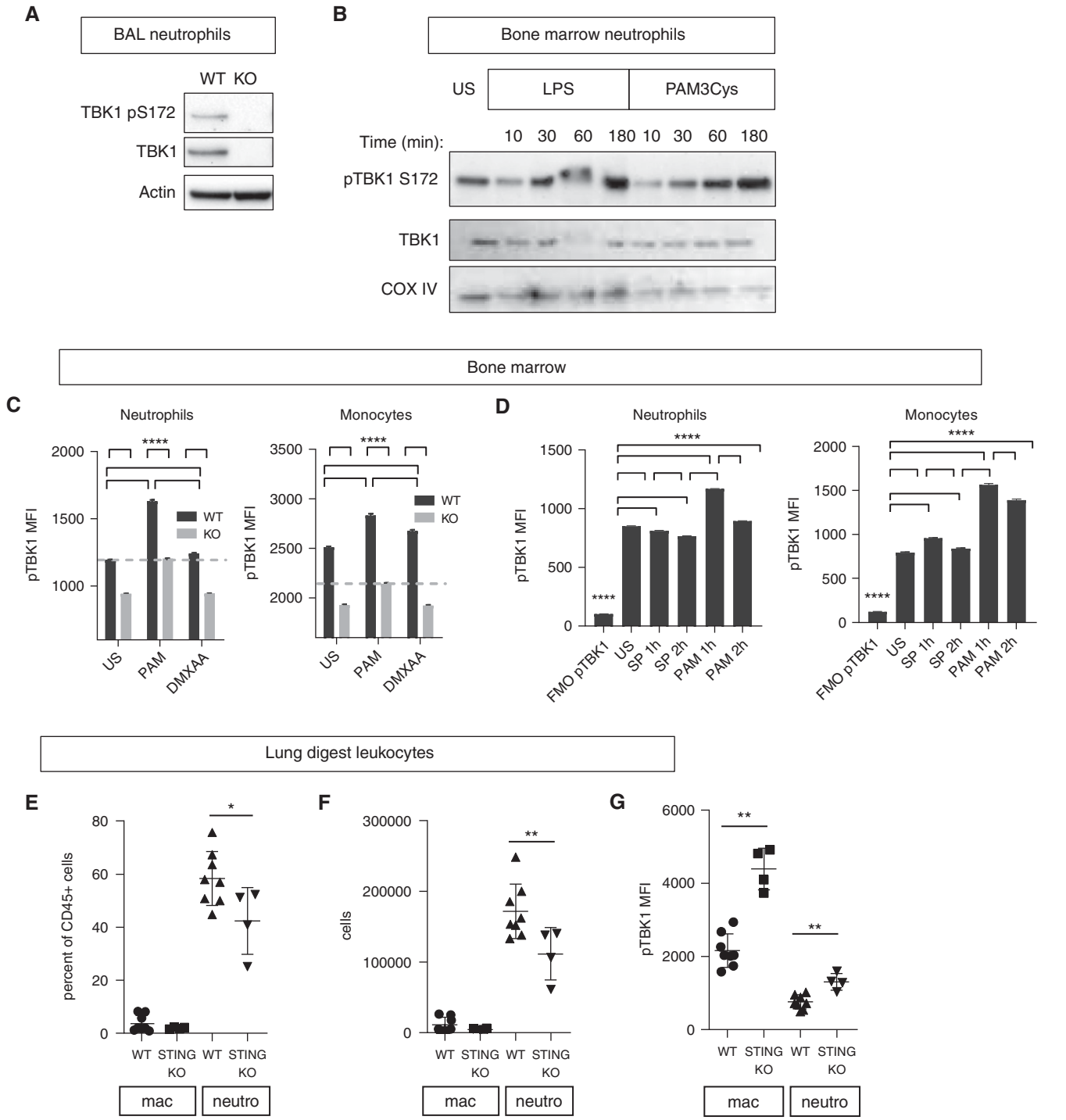

Figure 3. TBK1 is activated in neutrophils by bacterial ligands and bacteria. (*A*) WT or TBK1 KO mice were infected intratracheally with *S. pneumoniae*. At 24 hours p.i., lungs were lavaged, and neutrophils were isolated using anti-Ly6G beads before immunoblotting. (*B*) Bone marrow neutrophils were isolated with anti-Ly6G beads and stimulated with LPS (1 μg/ml) or PAM3Cys (10 μg/ml) for the indicated times before lysis and immunoblotting. (*C*) WT or TBK1 KO bone marrow was isolated and stimulated *in vitro* with PAM3Cys or DMXAA (25 μg/ml) for 2 hours and then permeabilized for intracellular staining of pS172 TBK1 and flow cytometry. Neutrophils were identified by Ly6G and monocytes by Ly6C expression. TBK1 KO cells were included to determine background staining or overlap staining from IKKε; the maximum background is depicted by the gray dashed line. (*D*) As in *C*, WT bone marrow was stimulated with *S. pneumoniae* or PAM3Cys for 1 or 2 hours *in vitro* and before flow cytometry for pTBK1. (*E–G*) Lung digests were prepared from STING KO and WT lungs at 24 hours after *S. pneumoniae* inoculation. Macrophages were defined as $CD45^+/CD64^+/Ly6G^-$ cells; neutrophils were defined as $CD45^+/CD64^-/Ly6G^+/CD11b^+$ cells. (*E*) The percentage of cells that were macrophages or neutrophils was determined. (*F*) The number of macrophages and neutrophils in the lungs was calculated. (*G*) Phosphorylation of TBK1 S172 was measured by intracellular staining in these lung digests from WT or STING KO mice. Immunoblots are representative of at least two independent experiments. $n = 4–8$ mice per group, with two independent replicates. $^*P < 0.05$, $^{**}P < 0.01$, and $^{****}P < 0.001$.

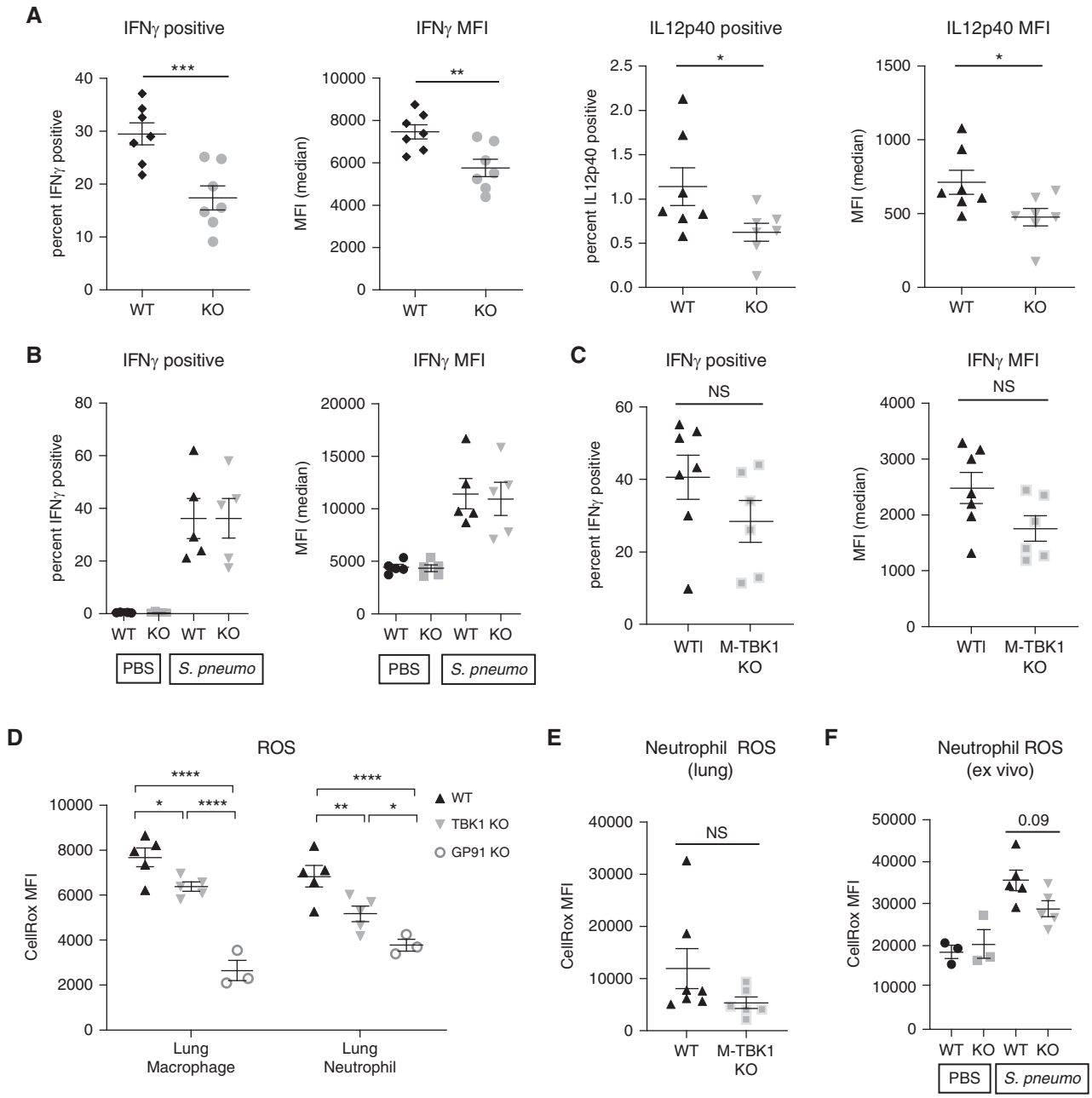

Figure 4. TBK1 is required for neutrophil IFNγ and reactive oxygen species (ROS) production. (*A*) Flow cytometry of neutrophils from lung digests of *S. pneumoniae*–infected WT and TBK1 KO mice at 24 hours p.i. showing the percentage expressing IFNγ or IL12p40 and the median fluorescence intensity (MFI). (*B*) Flow cytometry of bone marrow neutrophils from WT or TBK1 KO mice infected *in vitro* with *S. pneumoniae* for 4 hours. Graphs depict percentage of neutrophils expressing IFNγ and MFI. (*C*) Flow cytometry of neutrophils from lung digests of *S. pneumoniae*–infected WT and M-TBK1 KO mice at 24 hours p.i. showing the percentage of neutrophils expressing IFNγ and the MFI. (*D*) ROS production was assessed by the MFI of CellRox Deep Red in $CD64^+$ macrophages and $Ly6G^+$ neutrophils from lung digests of WT and TBK1 KO mice and (*E*) WT and M-TBK KO mice at 24 hours p.i. (*F*) ROS production by bone marrow neutrophils from WT or TBK1 KO mice treated with PBS or live *S. pneumoniae in vitro* for 2 hours. $n = 3$–8 mice per group, with two to three independent replicates. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, and $^{****}P < 0.001$.

expression by nontranscriptional means. Of note, our previous work has shown that ROS production is required for neutrophil IFNγ expression in *S. pneumoniae* infection (10), and as IFNγ is required for bacterial killing, the TBK1-ROS-IFNγ axis may represent a neutrophil-specific use of the TBK1 machinery.

In summary, we show that TBK1 is a critical component of the response to *S. pneumoniae* pneumonia and that deletion of TBK1 impairs multiple host defense functions in a manner distinct from deletion of the type I IFN pathway. At the cellular level, TBK1 is required for ROS and cytokine

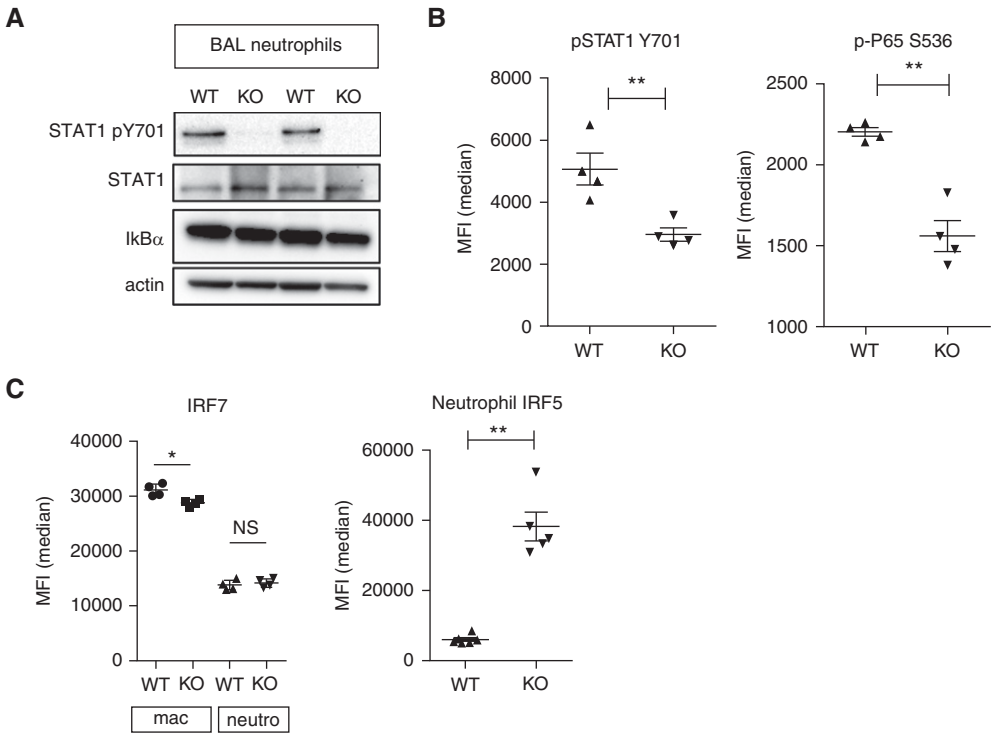

Figure 5. TBK1 is required for regulation of neutrophil transcription factors. (*A*) BAL neutrophils from infected mice were isolated with anti-Ly6G beads 24 hours p.i. and subjected to lysis and immunoblotting. (*B*) Phosphorylation of STAT1 Y701 and RelA/p65 S536 was measured by intracellular staining in neutrophils from lung digests using flow cytometry. (*C*) Amount of IRF7 and IRF5 expression in neutrophils and macrophages was assessed by intracellular flow cytometry. $n = 4$–6 mice per group, with two independent replicates. $^{*}P < 0.05$ and $^{**}P < 0.01$.

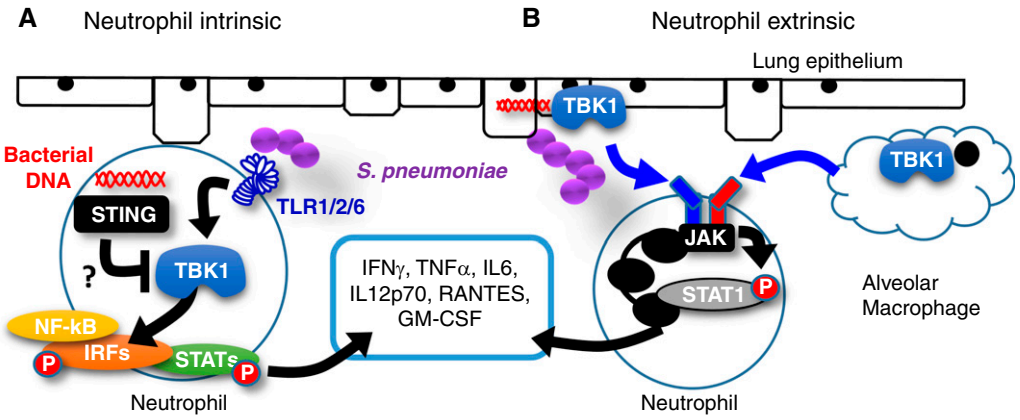

Figure 6. Models of TBK1 function in *S. pneumoniae* pneumonia. (*A*) Neutrophil-intrinsic model: pattern recognition receptors such as the TLR2 complex activate neutrophil TBK1 to phosphorylate transcription factors from the IRF, NF-κB, or STAT family, leading to expression of cytokines and ROS production. (*B*) Neutrophil-extrinsic model: TBK1 in epithelium or alveolar macrophages drives expression of type I and II IFNs and other cytokines, which activate neutrophil receptors, leading to JAK/STAT activation and cytokine expression.

production and for phosphorylation of neutrophil NF-κB and STAT1. At the organism level, TBK1 contributes to bacterial clearance and animal survival. These findings point to as-yet undiscovered connections between the noncanonical IKK family and antibacterial defense. TBK1 is a critical component of STING-independent antibacterial responses in the lung, and TBK1 is necessary for multiple neutrophil functions. These studies illuminate the functions of TBK1 in different myeloid populations and the uniqueness of host defense mechanisms in bacterial compared with viral infections. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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