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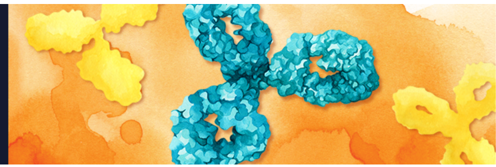

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Intracellular Bacterial Infection-Induced IFN-γ Is Critically but Not Solely Dependent on Toll-Like Receptor 4-Myeloid Differentiation Factor 88-IFN-$\alpha\beta$-STAT1 Signaling[1]

Antonio Gigliotti Rothfuchs, Christian Trumstedt, Hans Wigzell, and Martin E. Rottenberg[2]

Infection of murine bone marrow-derived macrophages (BMMϕ) with *Chlamydia pneumoniae* induces IFN-$\alpha\beta$-dependent IFN-γ secretion that leads to control of the intracellular bacterial growth. Enhanced growth of *C. pneumoniae* in Toll-like receptor (TLR) $4^{-/-}$ and myeloid differentiation factor (MyD) $88^{-/-}$ (but not TLR$2^{-/-}$, TLR$6^{-/-}$, or TLR$9^{-/-}$) BMMϕ is shown in this study. Reduced accumulation of IFN-α and IFN-γ mRNA was also observed in TLR$4^{-/-}$- and MyD$88^{-/-}$-infected cells. IL-1R and IL-18R signaling did not account for differences between MyD$88^{-/-}$ and wild-type BMMϕ. Surprisingly, infection-induced NF-κB activation as well as TNF-α, IL-1, or IL-6 mRNA expression were all normal in TLR$4^{-/-}$ and MyD$88^{-/-}$ cells. Phosphorylation of the transcription factor STAT1 during bacterial infection is IFN-$\alpha\beta$ dependent, and necessary for increased IFN-γ mRNA accumulation and chlamydial growth control. Signaling through common cytokine receptor γ-chain and RNA-dependent protein kinase both mediated IFN-$\alpha\beta$-dependent enhancement of IFN-γ mRNA levels. Accumulation of IFN-γ mRNA and control of *C. pneumoniae* growth required NF-κB activation. Such NF-κB activation was independent of IFN-$\alpha\beta$, STAT1, and RNA-dependent protein kinase. In summary, *C. pneumoniae*-induced IFN-γ expression in BMMϕ is controlled by a TLR4-MyD88-IFN-$\alpha\beta$-STAT1-dependent pathway, as well as by a TLR4-independent pathway leading to NF-κB activation. *The Journal of Immunology,* **2004, 172: 6345–6353.**

Mammalian Toll-like receptors (TLRs)[3] constitute a family of closely related transmembrane, primary signal-transducing proteins that respond to an array of microbial products (1). They recognize different pathogen-associated molecular patterns such as LPS, flagellin, unmethylated CpG motifs in DNA, dsRNA, mycobacterial lipoarabinomannan, yeast zymosan, and bacterial lipoproteins (1). Upon pathogen-associated molecular pattern recognition, the intracellular domains of all known TLRs interact with the adaptor molecule myeloid differentiation factor (MyD) 88 and initiate a common signaling cascade that leads to nuclear translocation of the transcription factors NF-κB and AP-1. This signaling cascade can also be activated upon IL-1 or IL-18R engagement (2). TLR can induce IFN-$\alpha\beta$ in the presence of MyD88 (3–5) or in its absence (6, 7) by using the adaptor named Toll-IL-1R domain-containing adaptor molecule/TIR domain-containing adaptor inducing IFN-β (8–10).

IFN-$\alpha\beta$ are key immunoregulatory cytokines produced directly after cell exposure to many pathogens. IFN-$\alpha\beta$ interfere with virus replication through induction of, e.g., double-stranded RNA-dependent protein kinase (PKR) and 2′-5′-oligoadenylate synthetase (2′5′OAS). Besides the antiviral effect exerted by inhibition of eukaryotic protein synthesis, PKR plays a catalytic or structural role to activate I-κB kinase or directly phosphorylate I-κB. Stress-activated protein kinases p38 and c-Jun kinase are also regulated by PKR in a pathway that leads to production of proinflammatory cytokines (11).

Binding of IFN-$\alpha\beta$ to their cellular receptor results in phosphorylation of STATs by receptor-associated Janus kinases, resulting in formation of homodimeric (STAT1 · STAT1), heterodimeric (STAT1 · STAT2), or heterotrimeric (STAT1 · STAT2 · IFN regulatory factor-9) protein complexes. These multimeric complexes translocate to the nucleus, where they bind to distinct DNA elements, leading to expression of IFN-inducible genes (12). The key macrophage-activating cytokine, IFN-γ, mediates resistance against intracellular bacteria and protozoa via activation of antimicrobial effector molecules (13). Although involved in different responses, both IFN-$\alpha\beta$R and IFN-γR signaling share in common STAT1. In turn, both IFNs have partially overlapping biological effects.

The obligate intracellular Gram-negative bacterium *Chlamydia pneumoniae* is a common cause of acute respiratory disease (14) and has been associated with development of atherosclerosis (15). *Chlamydia* are internalized by macrophages, but by avoiding phagolysosomal fusion are able to replicate intracellularly. IFN-γ is central in resistance to this pathogen both in vivo and in vitro (reviewed in Ref. 16). Several studies show that, besides NK and T cells, myeloid cells such as macrophages, dendritic cells (DCs), and neutrophils can also express IFN-γ (17). In accordance, we have shown that mouse bone marrow-derived macrophages (BMMϕ) infected in vitro with *C. pneumoniae* express IFN-γ that

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[3] Abbreviations used in this paper: TLR, Toll-like receptor; 2-AP, 2-aminopurine; BMMϕ, bone marrow-derived macrophage; DC, dendritic cell; γ_cR, common cytokine receptor $\gamma\alpha\mu\mu\alpha$-chain; ICE, IL-1-converting enzyme; IFU, inclusion-forming unit; iNOS, inducible NO synthase; MAPK, mitogen-activated protein kinase; MyD88, myeloid differentiation factor 88; 2′5′OAS, 2′-5′-oligoadenylate synthetase; PKR, RNA-dependent protein kinase; RAG, recombination-activating gene; SPG, sucrose-phosphate-glutamate; WT, wild type.

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Table I. *PCR primers used in this study*

Sequence	Sense Primer (5′-3′)	Antisense Primer (5′-3′)
iNOS	CCC TTC CGA AGT TTC TGG CAG CAG CAG C	GGC TGT CAG AGC CTC GTG GCT TTG G
IFN-α[a]	GAC TCA TCT GCT GCT TGG AAT GCA ACC CTC	GAC TCA CTC CTT CTC CTC ACT CAG TCT TGC C
IFN-γ	TCG ATC TTG GCT TTG CAG CTC TTC CTC ATG GC	TGC ACC TGT GGG TTG TTG ACC TCA AAC TTG GC
IL-1α	ATG GCC AAA GTT CCT GAC TTG TTT	CCT TCA GCA ACA CGG GCT GGT C
IL-6	ATG AAG TTC CTC TCT GCA AGA GAC T	CAC TAG GTT TGC CGA GTA GAT CTC
IL-15	CAT ATG GAA TCC AAC TGG ATA GAT GTA AGA TA	CAT ATG CTC GAG GGA CGT GTT GAT GAA CAT
TNF-α	GAT CTC AAA GAC AAC CAA CTA GTG	CTC CAG CTG GAA GAC TCC TCC CAG
PKR	TCC TCT GCC GTG GTT TTC CTT T	ACA GGA GCC TGC CTT TCT CTT T
2′5′ OAS	TGG CTG AAG AGG CTG ATG TGT G	TGA GGA AGG CTG GCT GTG ATT G
β-Actin	GTG GGC CGC TCT AGG CAC CAA	CTC TTT GAT GTC ACG CAC GAT TTC

[a] The IFN-α primer sequences recognize a consensus sequence present in IFN-α1, α2, and -α7.

in turn protects these cells against chlamydial growth. This IFN-γ production was IL-12 independent, but required IFN-αβ (18).

DCs, smooth muscle cells, macrophages, endothelial cells, and PBMCs are all activated by chlamydial infection or acellular chlamydial components in a TLR2- or TLR4-dependent way (19–22). However, further details of such activation, in particular with regards to IFN-γ ϵ expression, are unknown.

IFN-αβ-dependent induction of IFN-γ in splenocytes and T cells has been suggested to be STAT4 dependent (23, 24). However, IFN-αβ might also activate IFN-γ in an IL-15-mediated way: Expression of IL-15 depends on presence of a functional IFN-αβR (25, 26), and IFN-γ secretion by splenic DCs and macrophages was markedly increased after treatment with IL-15 (27). In this study, we have explored how *C. pneumoniae* recognition and the ensuing signaling pathways lead to protective IFN-γ expression in BMMϕ.

Materials and Methods

Mice

Mutant mouse strains without IFN-αβR (28), IFN-γR (29), STAT1 (30, 31), MyD88 (32), TLR2 (33), TLR4 (34), TLR6 (35), TLR9 (36), IL-1-converting enzyme (ICE) (37), common cytokine receptor chain (γ_cR) (38), or recombination-activating gene (RAG)-1 (39) were generated by homologous recombination in embryonic stem cells. RAG-1$^{-/-}$/γ_cR$^{-/-}$ mice were purchased from Taconic Farms (Germantown, NY). Animals were bred and kept under specific pathogen-free conditions. Mice of the C57BL/6 background were used as controls for IFN-γR$^{-/-}$, IFN-β$^{-/-}$, STAT1$^{-/-}$, MyD88$^{-/-}$, TLR2$^{-/-}$, TLR4$^{-/-}$, TLR6$^{-/-}$, TLR9$^{-/-}$, and ICE$^{-/-}$ mice, and 129Sv/Ev mice as controls for IFN-αβR$^{-/-}$ mice.

Generation of mouse BMMϕ

Mouse BMMϕ were obtained from 6- to 10-wk-old mice, as described (18). Mice were euthanized, and the femur and tibia of the hind legs were dissected. Bone marrow cavities were flushed with 5 ml of cold, sterile PBS. The bone marrow cells were washed and resuspended in DMEM (Sigma-Aldrich, St. Louis, MO) containing glucose and supplemented with 2 mM L-glutamine, 10% FCS, 10 mM HEPES, 100 μg/ml streptomycin, 100 U/ml penicillin (all from Sigma-Aldrich), and 20–30% L929 cell-conditioned medium (as a source of M-CSF). Bone marrow cells were passed through a 100-μm cell strainer, plated in six-well plates (1.2×10^7 cells/well, 2×10^6 cells/ml), and incubated for 7 days at 37°C, 5% CO_2. Before use, BMMϕ cultures were washed vigorously to remove nonadherent cells. Cells from several wells were also harvested and counted by trypan blue exclusion. Typically, bone marrow cells from one mouse yielded 2–3 × 10^6 BMMϕ after 7 days in culture. We have previously shown by immunofluorescence staining that these BMMϕ are F4/80$^+$, CD14$^+$, and Mac-3$^+$ (18).

Generation of fibroblasts from mouse lung

Primary fibroblast cultures were generated, as described (40). The pulmonary vasculature was perfused, and lungs were aseptically removed, excised into small pieces, and subjected to collagenase digestion at 37°C for 15 min under agitation. The resulting cell suspension was collected. Enzymatic digestion and collection of cell suspensions were repeated twice. Cell suspensions were then pooled, passed through a 100-μm cell strainer, washed, and plated in tissue culture flasks with IMDM supplemented with 2 mM L-glutamine, 5% FCS, 10 mM HEPES, 100 μg/ml streptomycin, and 100 U/ml penicillin at 37°C, 5% CO_2. Fresh medium was added every 7 days until fibroblast cultures attained confluence, ~7–14 days later. Lung primary fibroblasts were then washed vigorously, trypsinated, and replated onto six-well plates (2.5×10^5 cells/well, 0.42×10^5 cells/ml).

Infection and infectivity assay

Mycoplasma-free *C. pneumoniae* isolate Kajaani 6 (41) was propagated in HEp-2 cells. Bacteria were stored in small aliquots in sucrose-phosphate-glutamate (SPG) solution at −70°C until further use. The infectivity as measured by inclusion-forming units (IFU) of bacterial preparation was determined in HEp-2 cells, as described below.

Cultures of BMMϕ were infected with *C. pneumoniae* by centrifugation for 1 h, 500 × *g* at 35°C. A multiplicity of infection of 3 was used for both

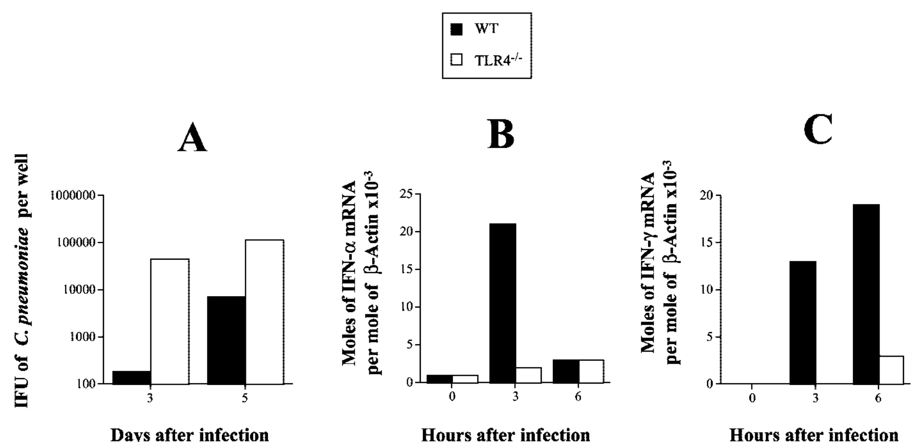

FIGURE 1. TLR4 is necessary for enhanced IFN-α and IFN-γ mRNA levels and controls growth of *C. pneumoniae* in BMMϕ. *A*, WT and TLR4$^{-/-}$ BMMϕ were infected with *C. pneumoniae* and lysed in SPG buffer at the indicated time points after infection. *C. pneumoniae* IFU in BMMϕ lysates were quantified by HEp-2 infectivity assay. A representative from three independent experiments is shown. *B* and *C*, Total RNA was extracted from WT and TLR4$^{-/-}$ BMMϕ at the indicated time points after infection with *C. pneumoniae*. Accumulation of IFN-α (*B*) and IFN-γ (*C*) mRNA was measured by competitive PCR. Similar results were obtained in two separate experiments.

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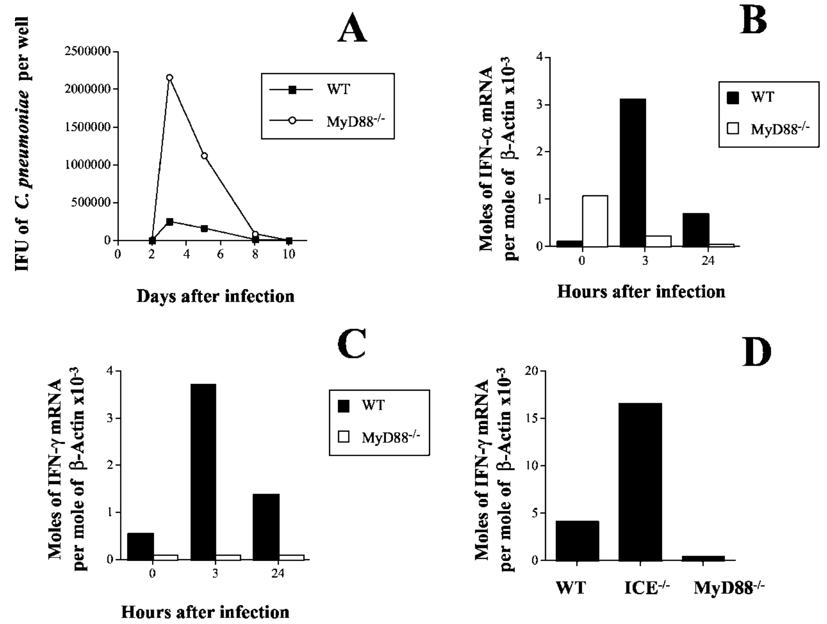

FIGURE 2. MyD88 is necessary for enhanced IFN-α and IFN-γ mRNA levels and controls growth of *C. pneumoniae* in BMMϕ. *A*, WT and MyD88$^{-/-}$ BMMϕ were infected with *C. pneumoniae* and lysed in SPG buffer at the indicated time points after infection. *C. pneumoniae* IFU in BMMϕ lysates were quantified by HEp-2 infectivity assay. For each time point shown, bacterial levels were found to be at least 6 times higher in MyD88$^{-/-}$ BMMϕ compared with WT BMMϕ. A representative from three independent experiments is shown. *B* and *C*, Total RNA was extracted from WT and MyD88$^{-/-}$ BMMϕ at the indicated time points after infection with *C. pneumoniae*. The accumulation of IFN-α (*B*) and IFN-γ (*C*) mRNA was measured by competitive PCR. Comparable results were obtained in two separate experiments. *D*, Total RNA was extracted from WT, MyD88$^{-/-}$, and ICE$^{-/-}$ BMMϕ at 6 h after infection with *C. pneumoniae*. The accumulation of IFN-γ mRNA was measured by competitive PCR. A representative from two independent experiments is shown.

BMMϕ and fibroblast experiments. At different time points after infection, cells were washed with PBS and then lysed in SPG buffer. Assessment of IFU in SPG lysates was done in HEp-2 cells. Aliquots of SPG lysates diluted 10- to 200-fold were used in duplicate to infect overnight cultures of confluent HEp-2 cells. The latter were grown in DMEM containing glucose and supplemented with 2 mM L-glutamine, 5% FCS, 10 mM HEPES, and 25 μg/ml streptomycin (DMEM/Strep) on round 13-mm^2 glass coverslides in 24-well plates. Inoculated cells were centrifuged for 1 h, 500 × *g* at 35°C. Thereafter, supernatant was removed and DMEM/Strep containing 0.5 μg/ml cycloheximide was added. Cells were incubated at 35°C for 72 h, 5% CO_2, thereafter washed gently with PBS, and fixed in methanol. Glass coverslides were then stained for 0.5 h at room temperature with a FITC-conjugated *Chlamydia* genus-specific mAb (1/5 dilution; Pathfinder *Chlamydia* Confirmation System; Bio-Rad, Hercules, CA). Coverslides were mounted with fluorescent mounting medium (DAKO, Carpinteria, CA), and IFU of *C. pneumoniae* were quantified by fluorescence microscopy. The infectivity was expressed as IFU of *C. pneumoniae* per well.

Competitive RT-PCR assay

Cultures of *C. pneumoniae*-infected BMMϕ were disrupted in RNAzol B (Nordic Biosite, Täby, Sweden), and total RNA was isolated according to the instructions of the manufacturer and reversed transcribed into cDNA, as described (42). Specific primer pairs for IFN-α, IFN-γ, inducible NO synthase (iNOS), PKR, 2′5′OAS, IL-1α, IL-6, IL-15, TNF-α, and β-actin were used to amplify cDNA. Amplified cDNAs were visualized in ethidium bromide-stained 2% agarose gels or quantified by competitive PCR assays. Briefly, competitor fragments with a different length, but using the same primers as the target cDNA, were constructed using composite primers and an exogenous DNA fragment (43). Three- to 4-fold serial dilutions of the competitor were amplified in the presence of a constant amount of cDNA. Reactions were conducted for 23–41 cycles in a thermal cycler (PerkinElmer, Cetus, CT) using an annealing step of 65°C for 2′5′OAS; 62°C for IL-15, 60°C for IFN-α, IFN-γ, iNOS, IL-1α, IL-6, and β-actin; and 54°C for PKR and TNF-α. All primer sequences are given in Table I.

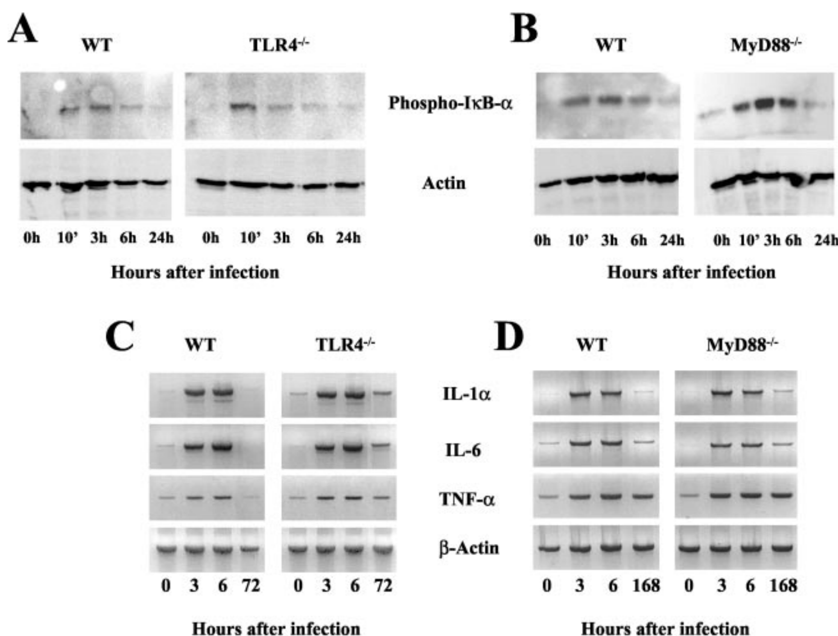

FIGURE 3. NF-κB activation in *C. pneumoniae*-infected BMMϕ does not require TLR4 and MyD88 signaling. *A* and *B*, WT (*A* and *B*), TLR4$^{-/-}$ (*A*), and MyD88$^{-/-}$ (*B*) BMMϕ were infected with *C. pneumoniae*. Protein extracts were prepared at the indicated time points after infection, separated by SDS-PAGE, electroblotted onto a nitrocellulose membranes, and immunoblotted with Abs that specifically recognize actin and phosphorylated I-κB-α. Abs were detected with HRP-conjugated anti-IgG, followed by ECL detection. *C* and *D*, Total RNA was extracted from WT (*C* and *D*), TLR4$^{-/-}$ (*C*), and MyD88$^{-/-}$ (*D*) BMMϕ at the indicated time points after infection with *C. pneumoniae*. The accumulation of IL-1α, IL-6, and TNF-α mRNA was visualized by RT-PCR. A representative from two independent experiments is shown.

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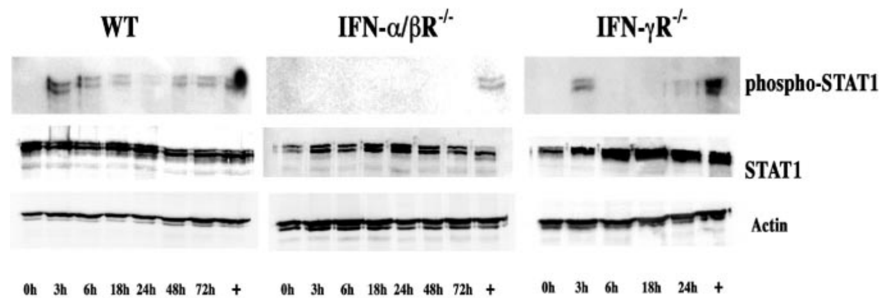

FIGURE 4. IFN-αβR and IFN-γR signaling completely account for STAT1 phosphorylation in *C. pneumoniae*-infected BMMϕ. WT, IFN-αβR$^{-/-}$, and IFN-γR$^{-/-}$ BMMϕ were infected with *C. pneumoniae*. Protein extracts were prepared at the indicated time points after infection and separated by SDS-PAGE, electroblotted onto nitrocellulose membranes, and immunoblotted with Abs that specifically recognize actin, total STAT1, and phosphorylated STAT1. Abs were detected with HRP-conjugated anti-IgG, followed by ECL detection. A representative from three independent experiments is shown.

SDS-PAGE and Western blotting

C. pneumoniae-infected BMMϕ were lysed in 150 mM NaCl, 20 mM Tris-HCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol, and 2 mM PMSF. The protein content in cell lysates was measured by Lowry assay (Bio-Rad). Sample buffer (Bio-Rad) containing 2-ME was added to samples that were then boiled for 5 min. A total of 10 μg of samples was separated at 100 V, 250 mA on 10% separating/5% stacking SDS-polyacrylamide gels. Samples were then transferred onto nitrocellulose membranes (Bio-Rad) by electroblotting at 100 V, 250 mA for 1 h. Immunostaining was performed using polyclonal rabbit anti-I-κB-α, monoclonal mouse anti-phosphorylated (Ser$^{32/36}$) I-κB-α, polyclonal rabbit anti-phosphorylated (Tyr^{701}) STAT1, polyclonal rabbit anti-STAT1 (all 1/1000 dilution; Cell Signaling, Beverly, MA), and polyclonal rabbit anti-actin (1/500 dilution; Sigma-Aldrich). Membranes were then washed and incubated with HRP-conjugated polyclonal rabbit anti-mouse Igs or HRP-conjugated polyclonal goat anti-rabbit Igs (both 1/2000 dilution; DAKO), developed using ECL-Plus (Amersham Pharmacia Biotech, Piscataway, NJ), and photographed using a Fuji intelligent darkbox II digital camera.

Results

TLR4 and MyD88 are necessary for enhanced IFN-α and IFN-γ mRNA levels and control growth of C. pneumoniae *in BMMϕ*

To investigate which TLR are essential for growth control of *C. pneumoniae*, TLR2$^{-/-}$, TLR4$^{-/-}$, TLR6$^{-/-}$, or TLR9$^{-/-}$ BMMϕ were infected with *C. pneumoniae*, respectively. Only TLR4$^{-/-}$ BMMϕ showed enhanced bacterial levels in comparison with wild-type (WT) controls (Fig. 1*A*). On the contrary, TLR2$^{-/-}$, TLR6$^{-/-}$, and TLR9$^{-/-}$ BMMϕ showed paradoxically lower *C. pneumoniae* levels than WT BMMϕ (data not shown), an observation that remains to be investigated. Enhanced chlamydial growth in TLR4$^{-/-}$ BMMϕ correlated with lack of enhanced IFN-α and IFN-γ mRNA accumulation (Fig. 1, *B* and *C*). Signaling through TLR4 occurs at least in part through MyD88. In line

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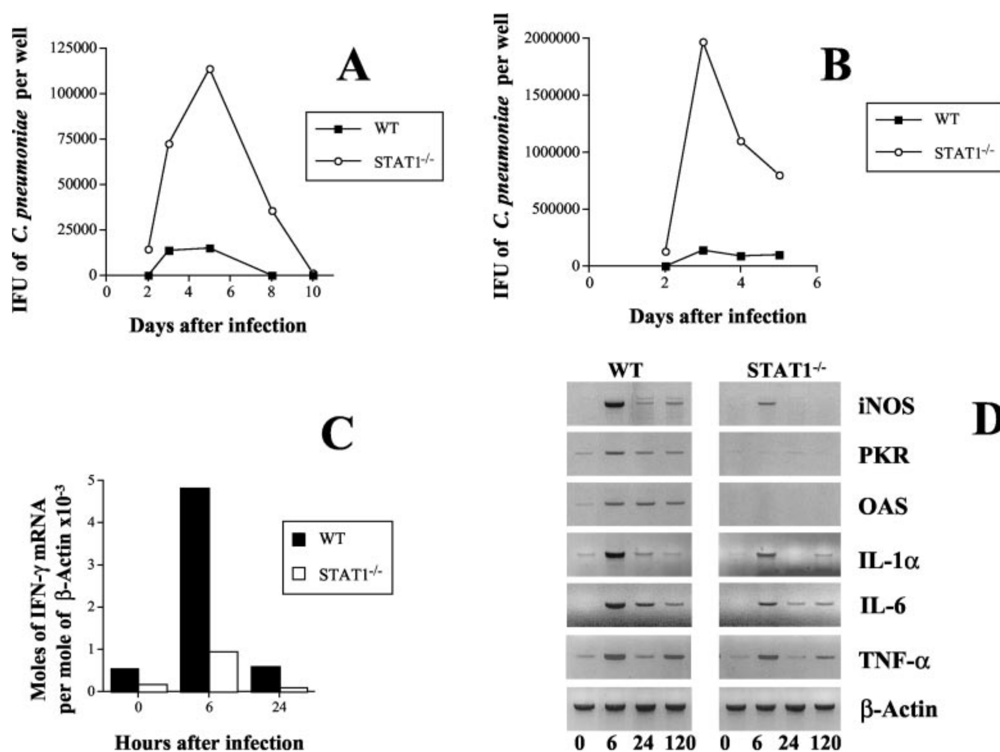

FIGURE 5. STAT1 is necessary for enhanced IFN-γ mRNA levels and controls growth of *C. pneumoniae* in BMMϕ. *A* and *B*, WT and STAT1$^{-/-}$ BMMϕ (*A*) and lung fibroblasts (*B*) were infected with *C. pneumoniae* and lysed in SPG buffer at the indicated time points after infection. *C. pneumoniae* IFU in the BMMϕ lysates were quantified by HEp-2 infectivity assay. For each time point shown, bacterial levels were found to be at least 5 times higher in STAT1$^{-/-}$ BMMϕ and 8 times higher in STAT1$^{-/-}$ fibroblasts compared with WT controls, respectively. A representative from three and two independent experiments for BMMϕ and fibroblasts, respectively, is shown. *C* and *D*, Total RNA was extracted from WT and STAT1$^{-/-}$ BMMϕ at the indicated time points after infection with *C. pneumoniae*. The accumulation of IFN-γ (*C*) mRNA was measured by competitive PCR, while that of iNOS, PKR, 2′5′OAS, IL-1α, IL-6, and TNF-α was visualized by RT-PCR (*D*).

with this, MyD88$^{-/-}$ BMMϕ were more susceptible to *C. pneumoniae* (Fig. 2*A*) compared with WT BMMϕ, and showed no increase in IFN-α and IFN-γ mRNA levels (Fig. 2, *B* and *C*). To confirm that the observations made in MyD88$^{-/-}$ BMMϕ indeed were linked to a defect in TLR signaling, ICE$^{-/-}$ BMMϕ were infected with *C. pneumoniae*. ICE is required for proteolytic activation of IL-1 and IL-18, which signal through MyD88 (44). ICE$^{-/-}$ BMMϕ showed nondiminished IFN-γ mRNA and *C. pneumoniae* levels compared with WT cells (Fig. 2*D*, and data not shown). Thus, reduced IFN-α and IFN-γ expression in infected MyD88$^{-/-}$ BMMϕ is due to a defect in TLR signaling.

Phosphorylation of I-κB-α is required before nuclear translocation of NF-κB, serving as marker of NF-κB activation (45, 46). Phosphorylation of I-κB-α increased in *C. pneumoniae*-infected WT BMMϕ (Fig. 3, *A* and *B*). Surprisingly, similar levels of phosphorylated I-κB-α were detected in infected TLR4$^{-/-}$, MyD88$^{-/-}$, and WT BMMϕ (Fig. 3, *A* and *B*). Levels of IL-1α, IL-6, and TNF-α mRNA in infected TLR4$^{-/-}$, MyD88$^{-/-}$, and WT BMMϕ were also similar (Fig. 3, *C* and *D*).

STAT1 is necessary for enhanced IFN-γ mRNA levels and controls growth of C. pneumoniae *in BMMϕ*

Biological effects of IFN-$\alpha\beta$ and IFN-γ are in part mediated via STAT1 (30). Phosphorylation of STAT1 is noted in *C. pneumoniae*-infected WT BMMϕ, but not in uninfected controls (Fig. 4). The level of phosphorylated STAT1 was relatively diminished in infected IFN-γR$^{-/-}$ and undetectable in IFN-$\alpha\beta$R$^{-/-}$ BMMϕ (Fig. 4). Growth of *C. pneumoniae* was higher (Fig. 5*A*) and IFN-γ mRNA accumulation lower (Fig. 5*C*) in STAT1$^{-/-}$ BMMϕ compared with WT controls. PKR, 2′5′OAS, and iNOS mRNA levels as well as NO in culture supernatants were all reduced in STAT1$^{-/-}$ BMMϕ, while mRNA levels of IL-1α, IL-6, and TNF-α were not or only slightly affected as compared with WT BMMϕ (Fig. 5*D*, and data not shown). Primary cultures of lung fibroblasts generated from STAT1$^{-/-}$ mice also displayed enhanced *C. pneumoniae* growth compared with WT controls (Fig. 5*B*).

Increased IFN-γ mRNA levels in C. pneumoniae*-infected BMMϕ are γ_cR dependent*

IL-15 signals through the γ_cR and can trigger release of IFN-γ from stimulated macrophages and DCs (25, 27). Enhanced IL-15 mRNA accumulation was observed in *C. pneumoniae*-infected WT and IFN-γR$^{-/-}$ BMMϕ, but not in IFN-$\alpha\beta$R$^{-/-}$ or STAT1$^{-/-}$ BMMϕ (Fig. 6*A*). RAG-1$^{-/-}$/γ_cR$^{-/-}$ BMMϕ showed higher *C. pneumoniae* numbers, whereas IFN-γ was reduced in comparison with infected RAG-1$^{-/-}$ BMMϕ (Fig. 6, *B* and *D*). RAG-1$^{-/-}$/γ_cR$^{-/-}$ and RAG-1$^{-/-}$ BMMϕ showed similar levels of IFN-α, IL-1α, IL-6, or TNF-α mRNA (Fig. 6, *C* and *E*).

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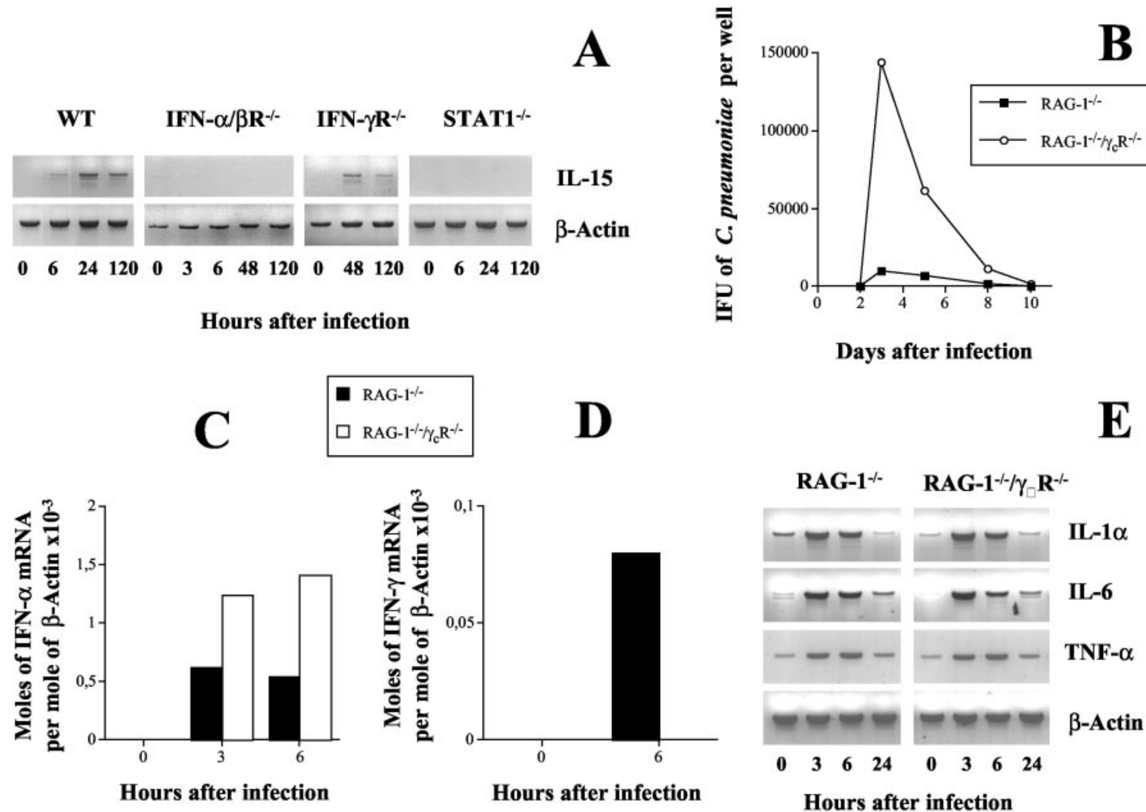

FIGURE 6. Increased IL-15 mRNA accumulation in *C. pneumoniae*-infected BMMϕ is dependent on signaling by IFN-$\alpha\beta$R and STAT1. *A*, Total RNA was extracted from WT, IFN-$\alpha\beta$R$^{-/-}$, IFN-γR$^{-/-}$, and STAT1$^{-/-}$ BMMϕ at the indicated time points after infection with *C. pneumoniae*. The accumulation of IL-15 was visualized by RT-PCR. For the sake of clarity, only the C57BL/6 WT control is represented, although similar results were obtained with 129Sv/Ev WT BMMϕ (data not shown). A representative from two independent experiments is shown. *B–E*, Increased IFN-γ mRNA accumulation in *C. pneumoniae*-infected BMMϕ is γ_cR dependent. RAG-1$^{-/-}$ and RAG-1$^{-/-}$/γ_cR$^{-/-}$ BMMϕ were infected with *C. pneumoniae* and lysed in SPG buffer at the indicated time points after infection. *C. pneumoniae* IFU in BMMϕ lysates were quantified by HEp-2 infectivity assay. For each time point shown, bacterial levels were found to be at least 5 times higher in RAG-1$^{-/-}$/γ_cR$^{-/-}$ BMMϕ compared with RAG-1$^{-/-}$ BMMϕ (*B*). Total RNA was extracted from RAG-1$^{-/-}$ and RAG-1$^{-/-}$/γ_cR$^{-/-}$ BMMϕ at the indicated time points after infection with *C. pneumoniae* (*C–E*). The accumulation of IFN-α (*C*) and IFN-γ (*D*) mRNA was measured by a competitive PCR, while IL-1α, IL-6, and TNF-α were visualized by RT-PCR (*E*). A representative from three independent experiments is shown.

PKR mediates IFN-αβ-dependent expression of IFN-γ mRNA

Enhanced PKR mRNA accumulation observed in *C. pneumoniae*-infected BMMϕ requires IFN-αβR and STAT1 (Fig. 5*D*, and data not shown). To investigate whether PKR was involved in IFN-αβ-dependent IFN-γ mRNA accumulation, 2-aminopurine (2-AP), a specific pharmacological inhibitor of PKR (47), was used. Treatment of WT, but not IFN-αβR$^{-/-}$ BMMϕ with 2-AP increased *C. pneumoniae* growth (Fig. 7*A*), suggesting that PKR acts downstream of IFN-αβR signaling. In line with this, *C. pneumoniae*-induced IFN-γ, but not IFN-α mRNA accumulation was dramatically reduced in 2-AP-treated WT BMMϕ (Fig. 7, *B* and *C*). These results suggest a positive role for PKR in IFN-γ expression. PKR can activate NF-κB through phosphorylation of I-κB-α or the IκB kinase complex components (11). However, treatment of BMMϕ with 2-AP did not affect IκB-α phosphorylation (Fig. 7*D*). Also, IFN-αβR$^{-/-}$ BMMϕ showed similar levels of phosphorylated I-κB-α as WT controls (Fig. 7*D*).

NF-κB activation is necessary for enhanced IFN-γ mRNA levels and control of C. pneumoniae *growth in BMMϕ*

We accordingly asked whether activation of NF-κB was linked to protection against *C. pneumoniae* infection of BMMϕ. For this purpose, BAY 11-7082, a pharmacological inhibitor of I-κB-α phosphorylation (48), was used. BAY 11-7082 treatment inhibited infection-induced I-κB-α phosphorylation in WT BMMϕ without affecting total I-κB-α levels (Fig. 8*B*). Treatment of *C. pneumoniae*-infected BMMϕ with BAY 11-7082 also increased chlamydial growth (Fig. 8*A*) and reduced IFN-γ (Fig. 9*A*) and iNOS (Fig. 9*B*), but had no impact on IFN-α mRNA accumulation (Fig. 9*C*). As expected, IL-1α, IL-6, and TNF-α levels were reduced following BAY 11-7082 treatment (Fig. 9*C*).

Discussion

We show in this study that macrophage infection with *C. pneumoniae* induces IFN-γ mRNA accumulation and bacterial growth control in a TLR4-MyD88-IFN-αβ-STAT1-dependent manner (Fig. 10). PKR and γ_cR signaling participate downstream of IFN-αβR in *C. pneumoniae* infection-induced IFN-γ expression and bacterial growth control. However, accumulation of IFN-γ mRNA does not solely depend on signals via TLR4-IFN-αβ pathway. NF-κB activation was observed in *C. pneumoniae*-infected macrophages and DCs (19, 21). We now show that NF-κB activation after *C. pneumoniae* infection is required for IFN-γ expression and chlamydial growth control, but can take place in a TLR4- and IFN-αβR-independent way. *C. pneumoniae*-induced proinflammatory cytokine transcripts are neither reduced in MyD88$^{-/-}$ nor TLR4$^{-/-}$ BMMϕ (Fig. 10).

In MyD88$^{-/-}$ macrophages, nuclear translocation of NF-κB and phosphorylation of the mitogen-activated protein kinases (MAPK) in response to LPS are somewhat delayed (32, 49). In contrast, TLR4$^{-/-}$ macrophages show no NF-κB and MAPK activation after exposure to LPS (34). Likewise, no activation of NF-κB or c-Jun kinase was observed in MyD88$^{-/-}$ macrophages in response to other TLR ligands such as peptidoglycan, lipoprotein, CpG DNA, or the imidazoquinolines (reviewed in Ref. 1).

Activation of NF-κB can, however, occur in a TLR4- and MyD88-independent manner in *C. pneumoniae*-infected BMMϕ. Thus, a TLR-independent signaling pathway probably also participates in bacterial induced NF-κB activation. It is also unlikely that other TLR mediate MyD88-independent proinflammatory cytokine expression (e.g., via Toll-IL-1R domain-containing adaptor molecule/TIR domain-containing adaptor inducing IFN-β) in our

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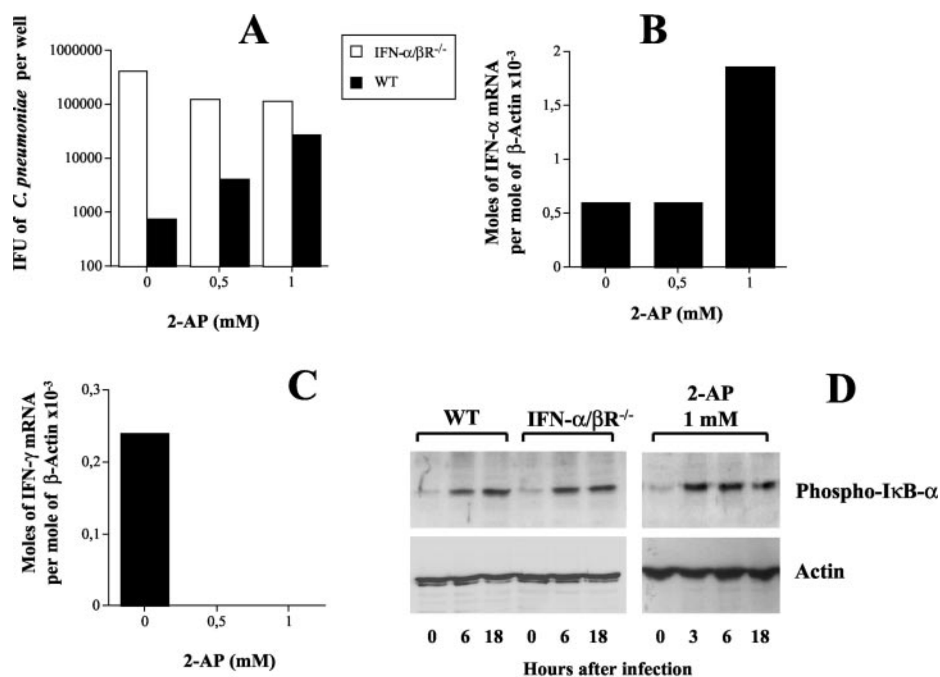

FIGURE 7. PKR mediates IFN-αβ-dependent expression of IFN-γ mRNA. *A*, WT and IFN-αβR$^{-/-}$ BMMϕ were treated with the PKR inhibitor 2-AP and infected with *C. pneumoniae*. Three days after infection, BMMϕ were lysed in SPG buffer, and *C. pneumoniae* IFU in BMMϕ lysates were quantified by HEp-2 infectivity assay. *B* and *C*, Total RNA was extracted from 2-AP-treated WT BMMϕ 6 h after infection with *C. pneumoniae*, and the accumulation of IFN-α (*B*) and IFN-γ (*C*) mRNA was measured by competitive PCR. A representative from two independent experiments is shown. *D*, WT, IFN-αβR$^{-/-}$, and 2-AP-treated WT BMMϕ were infected with *C. pneumoniae*. Protein extracts were prepared at the indicated time points after infection, separated by SDS-PAGE, electroblotted onto nitrocellulose membranes, and immunoblotted with Abs that specifically recognize actin and phosphorylated I-κB-α. Abs were detected with HRP-conjugated anti-IgG, followed by ECL detection. A representative from three independent experiments for IFN-αβR$^{-/-}$ BMMϕ and from two independent experiments for 2-AP-treated BMMϕ is shown.

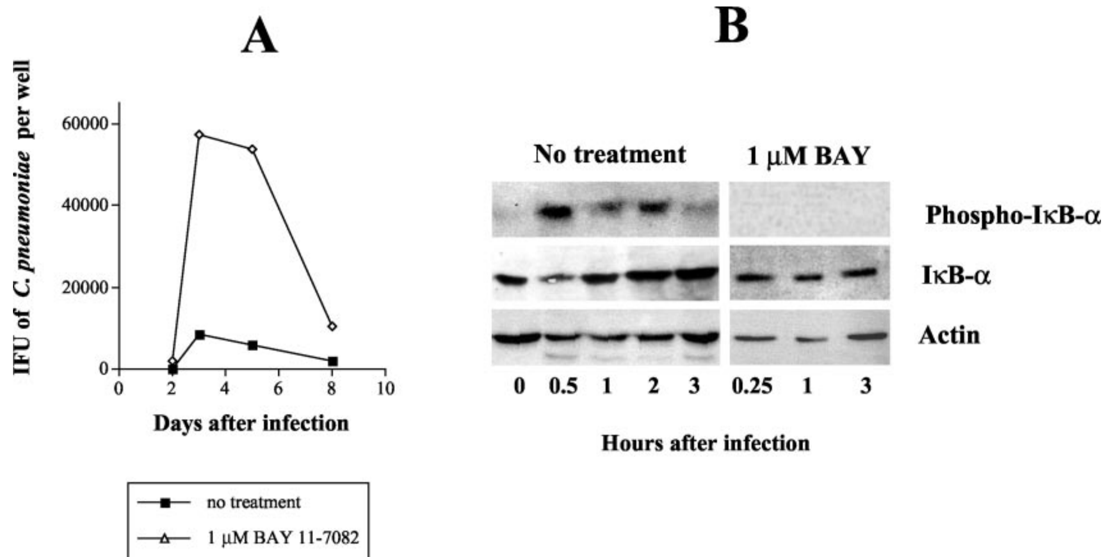

FIGURE 8. NF-κB activation is necessary for control of *C. pneumoniae* growth in BMMϕ. *A*, WT BMMϕ, treated with 1 μM BAY 11-7082 (4-methylphenyl)sulfonyl-2-properinitrite; Calbiochem, La Jolla, CA), solubilized in DMSO or left untreated, were infected with *C. pneumoniae*. Cells were lysed in SPG buffer at the indicated time points after infection. *C. pneumoniae* IFU in BMMϕ lysates were quantified by HEp-2 infectivity assay. For each time point shown, bacterial levels were found to be at least 5 times higher in BAY 11-7082-treated BMMϕ compared with untreated BMMϕ. A representative from two independent experiments is shown. *B*, WT BMMϕ were treated with 1 μM BAY 11-7082 and infected with *C. pneumoniae*. Protein extracts were prepared at the indicated time points after infection, separated by SDS-PAGE, electroblotted onto nitrocellulose membranes, and immunoblotted with Abs that specifically recognize actin and phosphorylated I-κB-α. Abs were detected with HRP-conjugated anti-IgG, followed by ECL detection.

experimental model, because expression of proinflammatory cytokines in response to different specific TLR ligands is all reduced in MyD88$^{-/-}$ cells (32, 49). However, TLR2 has been shown to be involved in TNF-α and IL-6 secretion in *Chlamydia trachomatis*-infected macrophages (50) and *C. pneumoniae*-infected DCs (19).

Activation of NF-κB is needed for IFN-γ expression, and thereby for growth control of *C. pneumoniae*. In line with this, NF-κB-binding elements have been identified in the IFN-γ promoter important for enhancement of gene transcription (51, 52). However, an IL-18-independent role for NF-κB in IFN-γ gene expression has not been previously reported.

Together, our results thus suggest that *Chlamydia* can induce both TLR-dependent and -independent pathways that cooperate for IFN-γ expression. TLR-analogous detection systems for microorganisms inside cells have been described (53). Nucleotide-binding oligomerization domain proteins in the cytoplasm are candidate among the molecules for such detection systems inside cells and recognize products from both Gram-positive and -negative bacteria (53–55). We plan to study the role for nucleotide-binding oligomerization domain proteins in IFN-γ secretion and chlamydial growth control.

In other system, listerial infection and LPS stimulation were shown to activate IFN regulatory factor 3 inducing the synthesis of IFN-β and IFN-α4 (7, 56). IFN regulatory factor 3 and IFN-αβ can be induced after activation of certain TLR in both MyD88-dependent and -independent ways (3, 4, 6, 10). However, MyD88-independent signaling was not sufficient for enhanced IFN-α mRNA accumulation in *C. pneumoniae*-infected BMMϕ.

Consistent with the importance of the Janus kinase-STAT pathway in mediating the actions of IFN-αβ, mice lacking either STAT1 (30, 31) or STAT2 (57) have impaired IFN-αβ-regulated responses and are highly sensitive to viral infection. However, IFN-αβ can also control cellular functions in a STAT1-independent fashion (26, 58–61): STAT1$^{-/-}$ mice are thus more resistant to virus infection than mice lacking expression of both IFN-αβR and IFN-γR (26, 58–61). We found that STAT1 was critical for the control of *C. pneumoniae* by BMMϕ. IFN-αβR signaling fully

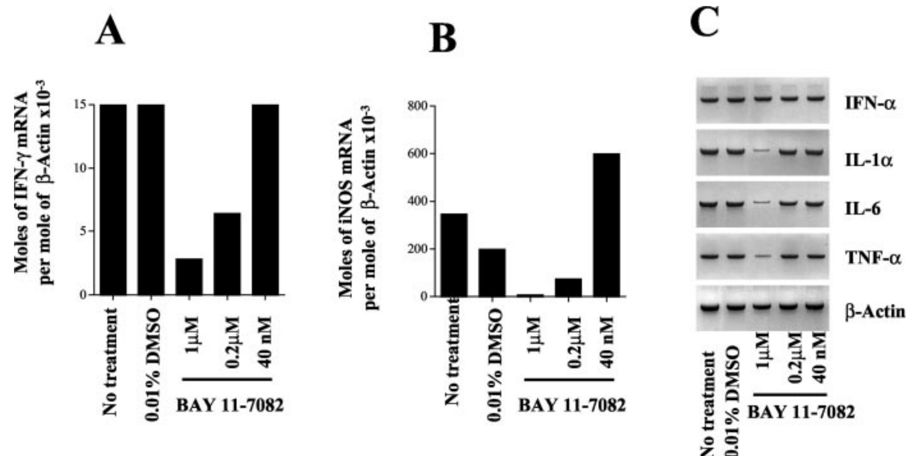

FIGURE 9. NF-κB activation is necessary for increased IFN-γ mRNA levels in *C. pneumoniae*-infected BMMϕ. *A–C*, Total RNA was obtained from WT BMMϕ treated with different concentrations of BAY 11-7082, 6 h after infection with *C. pneumoniae*. The accumulation of IFN-γ (*A*) and iNOS (*B*) mRNA was measured by competitive PCR, while that of IFN-α, IL-1α, IL-6, and TNF-α was visualized by RT-PCR (*C*). Similar results were obtained in two separate experiments.

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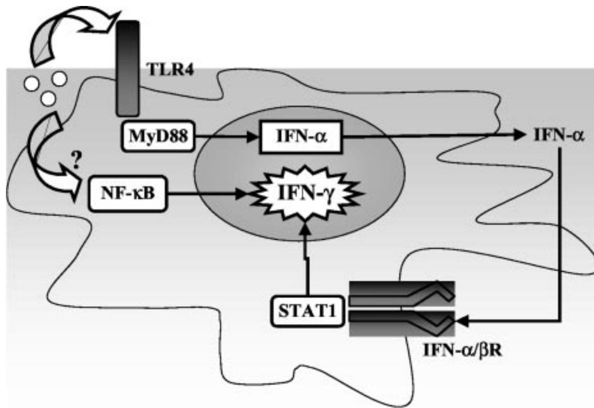

FIGURE 10. Molecular pathways controlling macrophage secretion of IFN-γ after infection with *C. pneumoniae*. BMMϕ infection with *C. pneumoniae* activates a TLR4-MyD88-dependent IFN-α expression. IFN-$\alpha\beta$-dependent, STAT1-mediated signaling is necessary for expression of IFN-γ. Activation of NF-κB, which can occur in a TLR4-MyD88- and IFN-$\alpha\beta$-independent way, is also critical for IFN-γ induction.

accounted for *C. pneumoniae*-induced STAT1 phosphorylation. Moreover, IFN-$\alpha\beta$-dependent STAT1 signaling was necessary for IFN-γ secretion. To our knowledge, our report is the first indicating the latter. Paradoxically, STAT4 activation was shown to be the critical intermediary in the induction of IFN-γ in IFN-$\alpha\beta$-stimulated T cells (24), while STAT1 was found to be a negative regulator of IFN-γ in the same system (62).

IL-15 is strongly induced by *C. pneumoniae* infection in WT or IFN-$\gamma R^{-/-}$, but absent in IFN-$\alpha\beta R^{-/-}$ or STAT1$^{-/-}$ BMMϕ. This is also occurring after influenza virus infection or LPS stimulation (26). *Chlamydia*-infected RAG-1$^{-/-}/\gamma_c R^{-/-}$ BMMϕ, which lack IL-15 responses, showed enhanced bacterial growth and decreased IFN-γ, but normal IFN-α mRNA accumulation.

PKR, responsible for IFN-$\alpha\beta$-dependent antiviral effects, also functions as a signal transducer in the proinflammatory response to many agents (11). Our results suggest that PKR is activated in *C. pneumoniae*-infected BMMϕ in an IFN-$\alpha\beta$-dependent manner and that it plays a role in IFN-γ expression and control of infection. However, such a protective role(s) of PKR (and IFN-$\alpha\beta$) does not depend on the NF-κB-activating properties of the enzyme. Whether PKR mediate control of chlamydial infection via MAPK activation remains to be explored.

In summary, our results indicate that TLR4-MyD88-dependent and TLR4-MyD88-independent signaling are both critical and complementary for IFN-γ expression in macrophages after intracellular bacterial infection. Our results indicate the simultaneous presence of high diversity and nonredundancy in the signals required for this process. A novel pathway of IFN-γ induction mediated by STAT1 activation is also demonstrated by our data.

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