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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-αβ-STAT1 Signaling

Antonio Gigiotti Rothfuchs, Christian Trumstedt, Hans Wigzell, and Martin E. Rottenberg

Infection of murine bone marrow-derived macrophages (BMMφ) with Chlamydia pneumoniae induces IFN-αβ-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 TLR2−/−, TLR6−/−, or TLR9−/−) BMMφ is shown in this study. Reduced accumulation of IFN-α and IFN-γ mRNA was also observed in TLR4−/− and MyD88−/−-infected cells. IL-1R and IL-18R signaling did not account for differences between MyD88−/− 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 TLR4−/− and MyD88−/− cells. Phosphorylation of the transcription factor STAT1 during bacterial infection is IFN-αβ 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-αβ-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-αβ, STAT1, and RNA-dependent protein kinase. In summary, C. pneumoniae-induced IFN-γ expression in BMMφ is controlled by a TLR4-MyD88-IFN-αβ-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) constitute a family of closely related transmembrane, primary signal-transducing proteins that respond to an array of microbial products. They recognize different pathogen-associated molecular patterns such as LPS, flagellin, unmethylated CpG motifs in DNA, dsRNA, mycobacterial lipoarabinomannan, yeast zymosan, and bacterial lipoproteins. Upon pathogen-associated molecular pattern recognition, the intracellular domains of all 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-18 engagement. TLR can induce IFN-αβ 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-αβ are key immunoregulatory cytokines produced directly after cell exposure to many pathogens. IFN-αβ 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-kB kinase or directly phosphorylate I-kB. 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-αβ 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-αβR 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.

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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/α, common cytokine receptor γ/α 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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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 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 upon 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 (cR) (30), RAG-1 (39), were generated by genetic means. MyD88 and IL-1R were also activated by homologous recombination in embryonic stem cells. RAG-1−/− 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 washed one or more times and replated onto six-well plates (2.5 × 10⁶ cells/well, 2 × 10⁶ cells/ml), and incubated for 7 days at 37°C, 5% CO₂. 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 × 10⁶ 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% CO₂. Fresh medium was added every 7 days until fibroblast cultures attained confluence, ~7–14 days later. Lung primary fibroblasts were then washed vigorously, trypsinized, and replated onto six-well plates (2.5 × 10³ cells/well, 0.42 × 10³ 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 −80°C. To use, cell suspensions were washed in 10% SPG medium and plated at 10⁶ CFU/ml onto HEp-2 cell monolayers in DMEM, 5% FCS, and 100 U/ml penicillin.

Table 1. PCR primers used in this study

| Sequence | Sense Primer (5′–3′) | Antisense Primer (5′–3′) |
|----------|----------------------|------------------------|
| iNOS     | CCC TTC CGA AGT TTC TGG CAG CAG C C | GGC TGT CAG AGC CTC GTG CTG TT |
| IFN-α    | GAC TCA TCT GCT GCT TGG AAT GCA ACC CTC | GAC TCA CTC CTT CTC CTC ACT CAG TGT C |
| IFN-γ    | TCG ATC TTT GCT TGG CAG CTC TTT AGT GC | TGG ACC TCA AAC AAC GAG T |
| IL-10    | ATG GCC AAA GTT CCT GAC TTG TT T | CCT TCA GCA ACA CGC GTG GTT C |
| IL-6     | ATG AAG TTT CTC TCT GCT ACA AGA T | CAC TAG GTT TGC GTA GAT CTC |
| IL-15    | CAT ATG GAA TAC 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 GTA | CTC CAG CTG GAA TAC CCC TCC CAG |
| PKR      | TCC TGC GCC GTG GTT TTC CTT TT | ACA GGA GCC TGC TTC TCT TT |
| 25S OAS  | TGG CAG AAG AGG GTG ATG TGT G | TCG TTA CAT GTG AGC CAT GAT TT |
| β-Actin  | GTC GGC CTC TGG AGC CAA A | CTC TTT GAT GTC AGC CAT GAT TT |

a The IFN-α primer sequences recognize a consensus sequence present in IFN-α1, α2, and α7.
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 by HEp-2 infectivity assay. For each time point shown, 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-mm2 glass coverslides in 24-well plates. Inoculated cells were centrifuged for 1 h, 500 X 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% CO2, 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 (1/50 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, Tiby, 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-lgG, 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.
FIGURE 4. IFN-αR and IFN-γR signaling completely account for STAT1 phosphorylation in C. pneumoniae-infected BMMφ. WT, IFN-αR$^{-/-}$, 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 for 1 h. Immunostaining was performed using polyclonal rabbit anti-IκB-α, monoclonal mouse anti-actin, total STAT1, and phosphorylated STAT1. Abs were formed using polyclonal rabbit anti-IκB-α, monoclonal mouse anti-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.

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. 1A). 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

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. 2A) 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. 2D, 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 IκB-α is required before nuclear translocation of NF-κB, serving as marker of NF-κB activation (45, 46). Phosphorylation of IκB-α increased in *C. pneumoniae*-infected WT BMMφ (Fig. 3, A and B). Surprisingly, similar levels of phosphorylated IκB-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-αβ 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-αβR−/− BMMφ (Fig. 4). Growth of *C. pneumoniae* was higher (Fig. 5A) and IFN-γ mRNA accumulation lower (Fig. 5C) 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. 5D, and data not shown). Primary cultures of lung fibroblasts generated from STAT1−/− mice also displayed enhanced *C. pneumoniae* growth compared with WT controls (Fig. 5B).

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

IL-15 signals through the γR 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-αβR−/− or STAT1−/− BMMφ (Fig. 6A). RAG-1−/−γR−/− 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−/−γR−/− and RAG-1−/− BMMφ showed similar levels of IFN-α, IL-1α, IL-6, or TNF-α mRNA (Fig. 6, C and E).

**FIGURE 6.** Increased IL-15 mRNA accumulation in *C. pneumoniae*-infected BMMφ is dependent on signaling by IFN-αβ and STAT1. A, Total RNA was extracted from WT, IFN-αβR−/−, 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 γR dependent. RAG-1−/− and RAG-1−/−γR−/− 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−/−γR−/− BMMφ compared with RAG-1−/− BMMφ (B). Total RNA was extracted from RAG-1−/− and RAG-1−/−γR−/− 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. 5D, 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. 7A), 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 IκB-α or the IκB kinase complex components (11). However, treatment of BMMφ with 2-AP did not affect IκB-α phosphorylation (Fig. 7D). Also, IFN-αβR−/− BMMφ showed similar levels of phosphorylated IκB-α as WT controls (Fig. 7D).

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 IκB-α phosphorylation (48), was used. BAY 11-7082 treatment inhibited infection-induced IκB-α phosphorylation in WT BMMφ without affecting total IκB-α levels (Fig. 8B). Treatment of C. pneumoniae-infected BMMφ with BAY 11-7082 also increased chlamydial growth (Fig. 8A) and reduced IFN-γ (Fig. 9A) and iNOS (Fig. 9B), but had no impact on IFN-α mRNA accumulation (Fig. 9C). As expected, IL-1α, IL-6, and TNF-α levels were reduced following BAY 11-7082 treatment (Fig. 9C).

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 γR 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- 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 model.
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-proprinirinitrite; Calbiochem, La Jolla, CA), solubilzed 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.

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.

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-proprinirinitrite; 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.

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-proprinirinitrite; 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 cyto-
kines in response to different specific TLR ligands is all reduced in
MyD88<sup>−/−</sup> 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 pro-
moter 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 oligomeriza-
tion domain proteins in the cytoplasm are candidate among the mol-
ecules 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
mRNA accumulation in C. pneumoniae-infected BMMφ.

Consistent with the importance of the Janus kinase-STAT path-
way 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-indepen-
dent fashion (26, 58–61): STAT1<sup>−/−</sup> 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
accounted for C. pneumoniae-induced STAT1 phosphorylation. Moreover, IFN-αβ-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-αβ-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-γR−/−, but absent in IFN-αβR−/− or STAT1−/− BMMφ. This is also occurring after influenza virus infection or LPS stimulation (26). Chlamydia-infected RAG-1−/−/γR−/− BMMφ, which lack IL-15 responses, showed enhanced bacterial growth and decreased IFN-γ, but normal IFN-α mRNA accumulation.

PKR, responsible for IFN-αβ-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-αβ-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-αβ) 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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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-αβ-dependent, STAT1-mediated signaling is necessary for expression of IFN-γ. Activation of NF-κB, which can occur in a TLR4-MyD88- and IFN-αβ-independent way, is also critical for IFN-γ induction.
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