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MyD88 Drives the IFN-β Response to *Lactobacillus acidophilus* in Dendritic Cells through a Mechanism Involving IRF1, IRF3, and IRF7

Gudrun Weiss,*† Kristina Maetoft-Udsen,* Sebastian A. Stifter,† Paul Hertzog,‡ Stanislas Goriely,‡ Allan R. Thomsen,§ Søren R. Paludan,§ and Hanne Frøkiaer*

Type I IFNs are induced by pathogens to protect the host from infection and boost the immune response. We have recently demonstrated that this IFN response is not restricted to pathogens, as the Gram-positive bacterium *Lactobacillus acidophilus*, a natural inhabitant of the intestine, induces high levels of IFN-β in dendritic cells. In the current study, we investigate the intracellular pathways involved in IFN-β upon stimulation of dendritic cells with *L. acidophilus* and reveal that this IFN-β induction requires phagosomal uptake and processing but bypasses the endosomal receptors TLR7 and TLR9. The IFN-β production is fully dependent on the TIR adapter molecule MyD88, partly dependent on IFN regulatory factor (IRF)1, but independent of the TIR domain-containing adapter inducing IFN-β MyD88 adapter-like, IRF and IRF7. However, our results suggest that IRF3 and IRF7 have complementary roles in IFN-β signaling. The IFN-β production is strongly impaired by inhibitors of spleen tyrosine kinase (Syk) and PI3K. Our results indicate that *L. acidophilus* induces IFN-β independently of the receptors typically used by bacteria, as it requires MyD88, Syk, and PI3K signaling and phagosomal processing to activate IRF1 and IRF3/IRF7 and thereby the release of IFN-β. *The Journal of Immunology*, 2012, 189: 2860–2868.

Type I IFNs are encoded by a multigene family comprising multiple α subtypes, IFN-β, and others which protect cells from viral infections and regulate both innate and adaptive immune responses (1, 2). Secreted type I IFN signals through a ubiquitously expressed two-chain receptor (IFNAR), thereby initiating a signaling cascade that triggers the expression of numerous anti-viral, anti-bacterial, and immunoregulatory IFN-stimulated genes (ISGs) involved in the innate host response (3). It is well established that cells exposed to type I IFNs display pro-nounced resistance to virus replication, as animals defective in IFN production are highly sensitive to virus infection (4). Type I IFNs have been demonstrated to contribute to the induction of a strong Th1 response via upregulation of IL-12 (5), activation of NK cells and various chemokines including the Th1 cell recruiting chemokine CXCL10 (6). IFN-β has also been recognized for its anti-inflammatory activity, as it inhibits the production of IL-1β (7), TNF-α, IFN-γ (8), and the neutrophil and leukocyte recruiting chemokine CXCL8 (9, 10). However, IFN-β can also be detrimental, as in models of sepsis LPS-induced IFN-β contributes to lethal toxicity (2).

Recognition of microbes by immune cells is mediated by pattern recognition receptors (PRRs), including TLRs and C-type lectins expressed on the surface of innate immune cells like monocytes, macrophages, neutrophils, and dendritic cells (DCs). PRRs recognize microbe-specific pathogen-associated molecular patterns and thereby activate several intracellular signaling pathways orchestrating both a cell type-specific and a pathogen-specific immune response in the host (11). In mice, 12 TLRs have been identified (12). TLR3, TLR7, TLR8, and TLR9 are localized in the endosomal compartments, whereas the other TLRs are associated with cell membranes. MyD88, an essential signaling component of all TLRs except TLR3, which utilizes TIR domain-containing adapter inducing IFN-β (TRIF), is required for activation of MAPKs and NF-κB leading to the production of proinflammatory cytokines such as IL-12, TNF-α, and IL-6 (13).

In recent years, evidence for bacterial induction of type I IFNs, in particular IFN-β, has emerged (14). Most of the bacteria tested belong to the Gram-negative genera, but a number of Gram-positive pathogenic bacteria are also able to activate IFN-β production in immune cells [reviewed by Monroe et al. (15) and Trinchieri (16)]. Gram-negative bacteria are primarily recognized by TLR4. Upon activation, TLR4 is endocytosed and delivered to intracellular vesicles where it forms complexes with TRAM and TRIF. Subsequently, TNFR-associated factor 3 (TRAF3) and the
protein kinases TBK1 and IKKi are recruited, and the IFN regulatory factor (IRF)3 is phosphorylated leading to the expression of type I IFN (17). Both TRIF and TRAM function in LPS–TLR4 signaling independently of MyD88 (18). In the case of Gram-positive bacteria, the majority of work has been conducted on the pathogenic bacteria Listeria monocytogenes and Streptococcus pyogenes. In macrophages, IFN-β induction by Group B Streptococcus as well as L. monocytogenes was completely abrogated in the absence of IRF3 but was unaffected in the absence of TLR3, TLR4, TLR7, TLR9, TRIF, TRAM, MyD88 adapter-like (Mal), MyD88, IRF1, or IRF7. By contrast, in DCs Group B Streptococcus-induced release of IFN-β was mainly dependent on TLR7, phagosomal degradation, MyD88, and IRF1 (19). In a recent study, S. pyogenes-induced IFN-β in DCs was dependent on MyD88, IRF3, and IRF5, whereas macrophages required IRF3 and partially MyD88 (20). Thus, bacteria can activate various cell type-specific pathways leading to the release of IFN-β. Notably, IFN-β induced by L. monocytogenes and S. pyogenes was reported to be dependent on IRF3 but independent of TLR2 (20-23).

We have previously shown that Lactobacillus acidophilus strains induce high amounts of IFN-β in DCs, which in turn leads to the expression of hundreds of ISGs and the activation of an innate immune response (24, 25). The capacity to induce IFN-β was markedly reduced in TLR2−/− DCs, dependent on clathrin-mediated endocytosis (24) and abrogated after blocking of the MAP JNK pathway (26). In this study, we show that L. acidophilus induces a strong IFN-β response in DCs, which subsequently drives an IFNAR1-dependent activation of the classical ISG Viperin. The L. acidophilus-triggered IFN-β production requires dynamin-dependent phagocytosis and maturation of phagolysosomes but is independent of the endosomal receptors TLR7 and TLR9. The IFN-β production is dependent on the TIR-containing adapter MyD88 but not TRIF or Mal and partially dependent on IRF1. Furthermore, our findings indicate that IRF3 or IRF7 are complementary in the induction of IFN-β. These characteristics of an IFN-β activation pathway have not been described previously.

Materials and Methods

Bacterial strains

The Gram-positive bacterium L. acidophilus NCFM (Danisco, Copenhagen, Denmark) was grown anaerobically overnight at 37°C in de Man Rogosa Sharp broth (Merck, Darmstadt, Germany). The Gram-negative bacterium Escherichia coli Nissle 1917 O6:K5:H1 (Statens Serum Institut, Copenhagen, Denmark) was grown aerobically overnight at 37°C in Luria-Bertani broth (Denmark) was grown anaerobically overnight at 37˚C in de Man Rogosa Sharp broth (Merck, Darmstadt, Germany) or Luria-Bertani broth (Danisco, Copenhagen, Denmark). Nissle 1917 O6:K5:H1 (Statens Serum Institut, Copenhagen, Denmark) was grown aerobically overnight at 37°C in Luria-Bertani broth (Denmark). The bacteria were subcultured twice, harvested by centrifugation at 1250 × g for 10 min, and washed twice in sterile PBS buffer.

Generation of DCs

Bone marrow-derived DCs were prepared from 6- to 10-wk-old mice. Cells were cultivated in RPMI 1640 with 10% heat-inactivated FCS in the presence of GM-CSF as described previously (27). Wild-type (WT) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). MyD88−/− and TLR9−/− mice were kindly provided by S. Akira (Osaka University, Osaka, Japan). Mal−/− were housed at Monash University (Clayton, Australia). IRF3−/− were obtained from T. Taniguchi (University of Tokyo, Tokyo, Japan). IRF3/−/− were bred at the Institute of Medical Immunology (Gosselies, Belgium), and IRF7−/− and IFNAR1−/− were housed at the Medical University of Copenhagen. All mice were used on a C57BL/6 background.

Stimulation of DCs with bacteria and treatments

Primary innate DCs (2 × 10⁵ cells/ml), tested >90% CD11c positive, were resuspended in fresh medium and seeded in 48-well tissue culture plates (500 µl/well) (Nunc, Roskilde, Denmark). Bacterial pellets were resuspended in antibiotic-free RPMI 1640 and added (100 µl/well) to the DCs at a multiplicity of infection (MOI) of 2:1. The TLR2 ligand lipoteichoic acid (LTA; InvivoGen, San Diego, CA) and the TLR4 ligand LPS (E. coli) (Sigma-Aldrich, Glostrup, Denmark) were added in final concentrations of 1 µg/ml. Polyribonucleosinic polyribocytidylic acid (Poly I:C) (InvivoGen), a synthetic analogue of dsRNA, was added in a final concentration of 10 µg/ml. The cell cultures were incubated at 37°C in 5% CO₂.

In the inhibitor experiments, DCs were preincubated for 30 min with BAY 61-3606 (Sigma-Aldrich), a highly selective inhibitor of the specific spleen tyrosine kinase (Syk), in final concentrations of 5 µM and 25 µM, 30 min with the Syk inhibitor picatannol (Sigma-Aldrich) in final concentrations of 5 µM and 25 µM, and 60 min with the PI3K inhibitor LY294002 (Sigma-Aldrich) in a final concentration of 50 µM. In the endosomal inhibitor experiments, DCs were pretreated with chloroquine (InvivoGen) in a final concentration of 10 µM; bafilomycin A1 (InvivoGen) in a final concentration of 25 µM; and Dynasore (Sigma-Aldrich) in a final concentration of 40 µM. Viability was verified by trypan blue staining.

Immunostaining and flow cytometry

DCs were harvested and resuspended in PBS supplemented with 1% (v/v) FBS and 0.15% (w/v) sodium azide containing anti-mouse FcγRII/III (3 µg/ml; BD Biosciences, San Jose, CA) to block nonspecific binding of Ab reagents. Allophycocyanin-conjugated anti-mouse CD86, FITC-conjugated anti-mouse CD11c (BD Pharmingen, San Diego, CA), and PE-conjugated anti-mouse CD40 (eBioscience, San Diego, CA) were used for staining. Nonspecific binding was evaluated by matched isotype controls for all Abs. DCs were analyzed using a BD FACSCanto II flow cytometer (BD BioSciences, San Jose, CA). Data analysis was performed using the software program FlowJo (Tree Star, Ashland, OR). The level of expression was measured as the geometric mean fluorescence intensity (MFI).

Cytokine quantification by ELISA

Cytokine production of was analyzed using the following commercially available ELISA kits: IL-12(p70), IL-10, TNF-α, and IL-6 (R&D Systems, Minneapolis, MN), and IFN-β (PBL Interferon Source, Piscataway, NJ).

RNA extraction

Murine bone marrow-derived DCs were harvested and total RNA was extracted using the MagMAX sample separation system (Applied Biosystems, Foster City, CA), including a DNase treatment step for genomic DNA removal. RNA quality was verified by Bioanalyzer (Agilent, Santa Clara, CA), and the concentration was determined by Nanodrop (Thermo, Wilmington, DE).

Quantitative real-time PCR analysis

Five hundred nanograms of total RNA was reverse transcribed by the TaqMan Reverse Transcription Reagent kit (Applied Biosystems) using random hexamer primers according to the manufacturer’s instructions. The obtained cDNA was stored in aliquots at −80°C. The expression of the genes encoding IFN-β, IL-12p40, and β-actin was detected using primers and probes as previously described (25). HPLC-purified forward and reverse primers were manufactured by DNA Technology (Aarhus, Denmark) and FAM-labeled probes by Applied Biosystems. A TaqMan Gene Expression Assay was purchased for the detection of Viperin (Rsd2) (Assay ID Mm00439546_s1; Applied Biosystems). The amplifications were carried out in a total volume of 10 µl containing 1× TaqMan Universal PCR Master Mix (Applied Biosystems) using the ABI Prism 7500 (Applied Biosystems) as previously described (25). The amplifications were normalized to the expression of the β-actin encoding gene. The samples generated in the Mal−/− experiments were analyzed with the 7900HT Fast Real-Time PCR System (Applied Biosystems). The TaqMan Gene Expression Assay for IFN-β (Assay ID Mm00439546_s1; Applied Biosystems), and were normalized to 18S (Assay ID 4319413E; Applied Biosystems). Amplification reactions were performed in triplicate, and DNA contamination controls were included. Relative transcript levels were calculated applying the 2(−ΔΔC(T)) method described by Livak and Schmittgen (28).

Type I IFN bioassay

Antiviral activity of IFNs was determined by inhibition of cytopathic effect of Semliki Forest Virus on L929 cells compared with a reference standard (GU-02-901-511) as described previously (29).

SDS-PAGE and Western blotting

DCs stimulated with L. acidophilus or E. coli were harvested by centrifugation and lysed in RIPA buffer (Thermo Fisher, Roskilde, Denmark) supplemented with protease and phosphatase inhibitor cocktails (P8340...
and P5726; Sigma-Aldrich) for 30 min on ice. After centrifugation for 20 min (4°C, 14,000 × g), the supernatant was stored at −20°C. For Western blotting, the samples were mixed with sample buffer (Invitrogen, Naerum, Denmark), boiled for 5 min, and resolved on 7% Tris acetate gels (Invitrogen) in Tris acetate SDS running buffer (Invitrogen) and transferred to Immun-Blot PVDF Membranes (Invitrogen) at 300 mA for 1 h. After blocking for 1 h in TBS containing 5% nonfat milk, membranes were washed three times in TBS and probed for 20 h with anti-Syk and anti–p-Syk (Tyr 525/526) (Cell Signaling) diluted in TBS. After extensive washing in TBS, membranes were incubated for 45 min with a secondary HRP-conjugated Ab (Dako, Glostrup, Denmark), and finally washed three times with TBS. The bound Abs were detected using ECL reagents according to the manufacturer’s instructions (GE Healthcare).

Statistical analysis

Statistical calculations were performed using the software program GraphPad Prism 5. One-way ANOVA and Bonferroni tests were applied (p < 0.01).

Results

L. acidophilus induces high levels of IFN-β and IL-12 in DCs

Bone marrow-derived DCs were stimulated with L. acidophilus, the dsRNA ligand Poly I:C, the TLR2 ligand LTA, the Gram-negative bacterium E. coli, and the TLR4 ligand LPS. Protein levels of IFN-β and IL-12 were measured in the supernatants after 10 h (Fig. 1A). L. acidophilus induced high amounts of IFN-β in DCs, as a protein expression of 2350 pg/ml was measured. For the RT-PCR analysis, cells were harvested after 2, 4, 6, 8, 10, and 16 h, RNA was extracted, and the gene expression was determined (Fig. 1B). E. coli, LPS, and Poly I:C induced Ifn-β ~50-fold at 2 h, which dropped at 4 h and remained very low. In contrast, Ifn-β was not detected at 2 h upon stimulation with L. acidophilus but was strongly induced at 4 h (420-fold), peaked at 6 h (510-fold), and was still expressed at high levels at 8 h and 10 h (380-fold and 290-fold, respectively). L. acidophilus also activated the IFN-β expression in bone marrow-derived macrophages; however, the protein expression was lower compared to DCs (data not shown). L. acidophilus likewise triggered a strong IL-12 response in DCs (Fig. 1C) with a gene expression profile of IL-12 similar to Ifn-β (Fig. 1D).

MyD88 is indispensable for the induction of IFN-β and IL-12 by L. acidophilus

TLR signaling involves either MyD88, which acts as an adapter molecule for TLR2, TLR4, TLR7, TLR8, and TLR9, or TRIF, which is the sole adapter molecule for TLR3 but also contributes to the IFN-β induction through TLR4 and IRF3 (11, 30). To investigate the involvement of the adapter proteins in the IFN-β response to L. acidophilus, we stimulated DCs derived from mice deficient for MyD88, TRIF, and IRF3 for 10 h. In contrast to the high levels of IFN-β in the supernatants of WT DCs, the IFN-β production was completely abolished in MyD88−/− DCs, whereas in TRIF−/− and IRF3−/− DCs, the IFN-β release was similar to the WT (Fig. 2A). In comparison, the induction of IFN-β in DCs stimulated with E. coli was reduced by 66% in the MyD88−/− DCs and completely absent in TRIF−/− and IRF3−/− DCs. In DCs stimulated with LPS, IFN-β was undetectable in MyD88−/−, TRIF−/−, and IRF3−/− DCs. IFN-β release by Poly I:C was slightly reduced in the MyD88−/− DCs (22%) and completely absent in TRIF−/− and IRF3−/− DCs. We also determined the expression of the Th1 skewing cytokine IL-12 as it is induced by IFN-β in an autocrine manner (5). Notably, the expression of IL-12 was completely absent in MyD88−/− cells upon L. acidophilus stimulation but increased by 35% in TRIF−/− and 86% in IRF3−/− DCs compared with WT cells (Fig. 2B). The induction of IL-12 upon addition of E. coli and LPS was significantly reduced in all three knockout mice tested, with no or very little IL-12 production in MyD88−/− and TRIF−/− DCs and an ~50% reduction in IRF3−/− DCs, which is in agreement with previous observations (31). Poly I:C did not trigger the expression of IL-12 in any of the cells tested.

**FIGURE 1.** L. acidophilus induces high levels of IFN-β and IL-12 in DCs. Bone marrow-derived DCs were stimulated with L. acidophilus (MOI 2:1), Poly I:C (10 µg/ml), LTA (1 µg/ml), E. coli (MOI 2:1), and LPS (1 µg/ml). (A and C) Production of IFN-β and IL-12 was measured by ELISA in supernatants after 10 h of stimulation. Data represent mean of measurements from triplicate cultures ± SD. *p < 0.01 (versus nonstimulated cells). (B and D) Expression of Ifn-β and Il-12 analyzed by RT-PCR and normalized to β-actin after 2, 4, 5, 8, 10, and 16 h of stimulation. Data presented are from one experiment representative of at least three independent experiments.
MyD88 is essential for the upregulation of the surface markers CD40 and CD86 in DCs stimulated with L. acidophilus.

To assess whether molecules required for costimulation of T cells are involved, we analyzed the upregulation of the maturation markers CD40 and CD86 by flow cytometry (Fig. 3). In WT DCs stimulated with L. acidophilus, CD40 and CD86 was expressed at a higher level than upon incubation with E. coli and Poly I:C. The induction of CD40 and CD86 upon stimulation with L. acidophilus was markedly impaired in the MyD88 \(^{-/-}\) cells, although not abrogated, whereas the induction by E. coli or Poly I:C was not affected. In the TRIF \(^{-/-}\) and IRF3 \(^{-/-}\) DCs, L. acidophilus upregulated CD40, which was absent upon Poly I:C stimulation. The
upregulation of CD86 was likewise markedly reduced in MyD88−/− DCs incubated with L. acidophilus but not with E. coli or Poly I:C, and in the TRIF−/− and IRF3−/− DCs only a slight upregulation was detected upon E. coli or Poly I:C stimulation. Thus, our data demonstrate that the adapter molecule MyD88 is dispensable for the L. acidophilus mediated upregulation of the surface maturation markers CD40 and CD86.

Induction of IFN-β by L. acidophilus in DCs does not require Mal

Mal acts to bridge MyD88 to TLR2 and TLR4 specifically via its TIR domain in response to bacterial infection (30, 32), but recent reports also describe a Mal-independent MyD88 pathway in the induction of the proinflammatory cytokines TNF-α and IL-6 (33, 34). We used DCs deficient for Mal to investigate whether this adapter protein is required for the induction of IFN-β by L. acidophilus. Surprisingly, coincubation of Mal−/− DC with L. acidophilus induced IFN-β protein levels more than three times compared with WT (Fig. 4A). This result was also confirmed by RT-PCR, as the Ifn-β expression was significantly higher after 6 and 8 h compared with WT (Fig. 4B). As our next step, we used a bioassay to test the antiviral property of the IFN released upon stimulation of WT and Mal−/− DCs with L. acidophilus (Fig. 4C). The protection measured was highest after 6 h in the WT and twice as pronounced in the supernatants of Mal−/− DCs. These results demonstrate that the adapter molecule Mal is not required for the induction of IFN-β by L. acidophilus in DCs.

L. acidophilus-induced IFN-β release requires phagosomal processing and Syk kinase signaling but not TLR7 and TLR9

To rework the rationale that IFN-β induction via MyD88-dependent and Mal- and IRF3-independent mechanisms implies endosomal TLRs, we tested whether phagosomal uptake, processing of L. acidophilus, and the endosomal receptors TLR7 and TLR9 are essential for the release of IFN-β. We prestimulated DCs with bafilomycin A1 and chloroquine, which are both inhibitors of endosomal acidification. To assess the importance of endosome maturation we included Dynasore, a selective noncompetitive GTPase inhibitor that blocks dynamin-dependent endocytosis and thereby the scission of endocytic vesicles. Pretreatment of DCs with chloroquine, bafilomycin A1, or Dynasore completely abolished the expression of Ifn-β upon addition of L. acidophilus to the cells (Fig. 5A). As these results indicate that endosomal acidification is essential for the induction of IFN-β, we investigated the involvement of the endosomal receptors TLR7 and TLR9. We stimulated DCs from WT, TLR7−/−, and TLR9−/− mice with L. acidophilus and measured the protein expression of IFN-β in the supernatants. Neither TLR7 nor TLR9 is required for IFN-β signaling, as the IFN-β levels expressed were in alignment with the WT (Fig. 5B).

We have previously shown that the MAP JNK pathway is mandatory for the expression of IFN-β in DCs stimulated with L. acidophilus (25, 26). We therefore sought to identify intermediate molecules upstream of the JNK pathway. As JNK phosphorylation is dependent on Syk signaling (35) and Syk collaborates with TLR receptors (36), we speculated that blockage of Syk might impair the IFN-β response to L. acidophilus. Indeed, pretreatment of DCs with the Syk inhibitors BAY 61-3606 and piceatannol prior to stimulation with L. acidophilus reduced the protein expression of IFN-β (Fig. 5C). By contrast, when E. coli was added to DCs preincubated with BAY 61-3606 and piceatannol, the expression of IFN-β only significantly decreased in the DCs pretreated with BAY 61-3606 but not with piceatannol. To verify the inhibitory potency of piceatannol, we measured the expression of IL-12 in DCs stimulated with L. acidophilus reduced the expression of IL-12 in the supernatants. Both Bay 61-3606 and piceatannol blocked the induction of IL-12 in DCs stimulated with L. acidophilus as well as E. coli (Supplemental Fig 1). We also pretreated DCs with LY294002, an inhibitor specific for PI3K. PI3K is a direct binding partner of Syk that participates in Syk-mediated downstream signaling and is also involved in phagocytosis (35). As anticipated,

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Induction of IFN-β by L. acidophilus in DCs does not require Mal. Bone marrow-derived DCs from WT and Mal−/− were stimulated with L. acidophilus (MOI 2:1). (A) Protein levels of IFN-β measured in the supernatants of WT and Mal−/− DCs by ELISA after 10 h. (B) Gene expression of IFN-β determined by RT-PCR over time in WT and Mal−/− DCs. (C) Biological activity of IFN measured via bioassay in the supernatants of WT and Mal−/− DCs over time. Data represent mean of measurements from triplicate cultures ± SD from three independent experiments. *p < 0.01 (versus WT).
The induction of Ifn-β was downregulated by 93% when PI3K was blocked via addition of LY294002 (Fig. 5D). To confirm our data, we analyzed the phosphorylation of Syk by Western blotting (Fig. 5E). Both L. acidophilus and E. coli activated Syk in DCs, but whereas E. coli-induced phosphorylation of Syk peaked at 15 min, stimulation with L. acidophilus resulted in a more pronounced expression that was first detected at 30 min of incubation. Thus, our data indicate that Syk signaling and phagosomal maturation of L. acidophilus is required for the induction of IFN-β.

L. acidophilus induced IFN-β signals independently of IRF3 and IRF7 but is reduced in IRF3/- and IRF1-deficient DCs

To further investigate the involvement of IRFs downstream of the MyD88 pathway, we used DCs deficient for IRF1, which is a positive regulator of the transcription of type I IFN genes (37). As we were able to show that IRF3 is not essential for the production of IFN-β, we sought to investigate the role of IRF7, a main regulator of the type I IFN induction (38). DCs from IRF1-/-, IRF3-/-, IRF7-/-, and IRF3/IRF7-/- mice were stimulated with L. acidophilus for 10 h (Fig. 6A). The protein levels of IFN-β were not affected by the absence of IRF3 or IRF7 but significantly reduced in double-deficient DCs suggesting complementary roles of IRF3 and IRF7 in regulating the gene expression of IFN-β. In IRF1-deficient DCs, the expression of IFN-β was significantly decreased (50%) compared with the WT. Autocrine IFN-β stimulation was confirmed, as the classical type I IFN-induced gene Viperin (Rsad2) (39) was not induced in IFNAR-/- DCs (Fig. 6B).

Our findings suggest that L. acidophilus activates several pathways in DCs leading to the induction of IFN-β involving the transcription factors IRF1, IRF3, and IRF7.

Discussion

The production of IFN-β by DCs and macrophages stimulated with pathogenic bacteria is well established. However, the levels induced are both bacteria- and cell-type dependent as differential responses have been observed (19, 20), which might be due to activation of diverging cellular signaling pathways. Importantly, the expression of IFN-β induced by L. acidophilus in DCs is
among the highest reported for bacteria able to induce a type I IFN response. In the current study, we aimed to identify receptors and adapter molecules involved in the IFN-β induction of this non-pathogenic Gram-positive bacterium. Our results clearly show that MyD88 is a prerequisite for the release of IFN-β, whereas absence of TRIF has no effect. Although it is well established that the Poly I:C pathway of IFN-β induction is MyD88 independent, we also observed a reduction of IFN-β in the MyD88−/− cells stimulated with this TLR3 ligand. However, in comparison with L. acidophilus, this effect is only subtle and may result from a general defect caused by knocking down the MyD88 gene. Notably, in the absence of Mal, a coadapter protein of MyD88 critical for TLR2 and TLR4 signaling (40, 41), the induction of IFN-β is significantly increased. Our data indicate that Mal not only is dispensable in MyD88 signaling but also might act as a repres-
s significantly increased. Our data indicate that Mal not only is
s in Mal−/− DCs, our findings are in accor-
dependent on MyD88 but independent of Mal. Furthermore, they
we observed a reduction of IFN-β in the MyD88−/− cells stimu-
ated with this TLR3 ligand. However, in comparison with L. aci-
ducible by an unidentified cytosolic receptor after rup-
location of IRF1 to the nucleus in DCs (47). IRF1 is essential for
the induction of several TLR-dependent genes; however, it remains
unclear how MyD88 activates IRF1. IRF7 resides in the cytoplasm
in an inactive form that has to be activated through PRR ligation
to induce type I IFN expression (48). Although the expression of
both genes was upregulated upon stimulation with L. acidophilus
in our recent study (24), the proteins may be present in the cell
prior to stimulation and thus play a role in the induction of IFN-β.
Our observation is supported by Dietrich et al. (49), who dem-
strated that in macrophages uptake and trafficking of TLR2 to
the endosomal compartments induced type I IFNs via MyD88-
dependent IRF1 and IRF7 pathways. Whether TLR2, which we
previously reported partly essential for the induction of IFN-β
in L. acidophilus-stimulated DCs (24), translocates to the endo-
somal compartments and thereby induces the release of IFN-β is
currently under investigation.

All TLRs, with the exception of TLR3, signal through MyD88
(32). As L. acidophilus requires MyD88, endocytosis, and acid-
ification of the phagosome to induce IFN-β, we stimulated DCs
deficient for the two endosomal nucleic acid sensors TLR7 and
TLR9. Several bacteria are reported to activate IFN-β via endo-
somal receptors (19, 20, 50). However, conflicting data exist whether
the Gram-positive Streptococcus induces IFN-β in DCs via TLR7
(19, 20). Our results reveal that TLR7 and TLR9 are not essential
for the induction of IFN-β by L. acidophilus in DCs; however, we
cannot exclude simultaneous activation of both receptors leading
to redundancy. The PRR responsible for triggering the MyD88–
IRF pathway therefore remains to be determined. Stimulation with
heat-killed but not UV-killed L. acidophilus induced a signifi-
cantly lower induction of IFN-β compared with live bacteria (data
not shown), thus indicating that an intact bacterium with native
molecules is a prerequisite for a strong expression of IFN-β.

The Gram-positive bacterium L. monocytogenes is primarily
detected by cytosolic sensing pathways due to its ability to escape
from phagosomes. L. monocytogenes is able to disrupt the phag-
osomal membrane via the pore-forming toxin listeriolysin O (51).
Listeriolysin O-deficient bacteria are trapped and unable to induce
type I IFN, whereas WT strains get released into the cytosol and
induce IFN-β dependent on IRF3 (23, 52, 53). Many bacteria are
reported to induce IFN-β through IRF3-dependent mechanisms
(20–22, 54). It has been proposed that the IRF3-dependent path-
way is activated by an unidentified cytosolic receptor after rup-
ture of the phagosomal membrane (22, 54), injection of bacterial
products into the cytosol (54), or translocation of digested bacterial
components from lysosomes into the cytosol (23). In the current
study, our results reveal that the IRF3-dependent pathway alone
is not a prerequisite for the induction of IFN-β by L. acidophilus
in DCs, which suggests that different activation mechanisms are
used. The expression of IFN-β was also not dependent on IRF7;
however, in the absence of both IRF3 and IRF7, IFN-β was sig-

FIGURE 6. L. acidophilus induced IFN-β signals independently of IRF3 and IRF7 but is reduced in
IRF3/7- and IRF1-deficient DCs. Bone marrow-de-
derived DCs from IRF-3−/−, IRF7−/−, IRF3−/−, IRF1−/−, and IFNAR1−/− mice were stimulated with L. aci-
dophilus (MOI 2:1). (A) Protein levels of IFN-β measured after 10-h incubation. (B) Gene ex-
pression of Viperin analyzed by RT-PCR in WT and
IFNAR1−/− DCs after 10-h incubation with L. aci-
dophilus (MOI 2:1). Data represent mean of measure-
ments from triplicate cultures (versus WT).
nificantly reduced. Thus, it is indicated that IRF3 and IRF7 have complementary roles and that various pathways are activated to trigger the release of IFN-β.

As mentioned above, translocation of whole bacteria and bacterial products appears to be due to degradation of the phagosomal membrane by secreted toxins, which form pores in the membrane and thereby enable the release of microbial components outside of the phagosomal compartment. The expression of cytolysins has been reported essential for the induction of IFN-β by Gram-positive bacteria (52, 54, 55). However, Group A Streptococcus is able to induce IFN signaling independently of cytolysis (21).

To date, pore-forming toxins, lysins, or other bacteria secretion systems have not been documented for L. acidophilus. It is worth noting that in contrast to the pathogenic bacteria L. monocytogenes and Group A Streptococcus strains, L. acidophilus is a commensal bacterium and a natural inhabitant of the intestine and therefore not considered pathogenic. Thus, it remains to be determined whether L. acidophilus uses similar mechanisms and whether various bacterial products such as DNA are able to escape the phagolysosomal compartment and are recognized by DNA cytosolic sensors (56–58). Our results reveal that the recognition of L. acidophilus by the host and a subsequent activation of the immune system strongly depend on functional phagocytosis and professional phagocytic cells able to engulf the bacterium presented. The ability of DCs to induce these high amounts of IFN-β upon stimulation with L. acidophilus lends itself well as a potential target for therapeutic application.

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Disclosures

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References