

Inhibition of dynamin-dependent endocytosis interferes with type III IFN expression in bacteria-infected human monocyte-derived DCs

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ABSTRACT

Type I IFNs (IFN- α/β s) and type III IFNs (IFN- λ 1-3) play an important role in host defense against viral infections. The induction of type I IFNs has recently been found to take place also in bacterial infections, and therefore, this study focuses on analyzing the regulation of type III IFNs in response to bacterial stimulation. We found by quantitative RT-PCR that the expression of IFN- λ 1 and IFN- λ 2/3 mRNAs, as well as that of IFN- β , was similarly up-regulated in response to stimulation with live *Salmonella typhimurium* or TLR4 agonist LPS in human moDCs. The induction of IFN- λ mRNAs did not require ongoing protein synthesis, and only IFN- λ 1 was detected at the protein level. The induction of IFN- λ mRNAs was sensitive to SB202190, Ly294002, and PDTC, which inhibit p38 MAPK, PI3K, and NF- κ B activation, respectively. Furthermore, we observed that blocking dynamin-dependent endocytosis pathways with dynasore led to decreased cell surface expression of CD86 and HLA class II molecules and reduced production of IFN- λ 1, CXCL10, and IL-6 when the cells were infected with *S. typhimurium*. Cytokine production was also impaired in dynasore-treated, *Streptococcus thermophilus*-stimulated cells. Further, inhibition of dynamin prevented *S. typhimurium*-induced phosphorylation of IRF3 and the internalization of the bacteria. In summary, induction of type III IFNs in bacteria-infected human moDCs requires multiple signaling pathways and involves bacterial phagocytosis. *J. Leukoc. Biol.* **88**: 665–674; 2010.

Abbreviations: CHX=cycloheximide, Ct=comparative threshold, h=human, IRF=IFN regulatory factor, ISRE=IFN-stimulated response element, mo=monocyte-derived, MxA=myxovirus-resistance protein A, NLR=nucleotide-binding oligomerization-like receptor, P-IRF3=phosphorylated IRF3, PDTC=pyrrolidine dithiocarbamate, RLR=retinoic acid-inducible gene I-like receptor, TIRAP=Toll/IL-1R-associated protein, TRAM=Toll-IL-1R domain-containing adaptor-inducing IFN- β -related adaptor molecule, TRIF=Toll-IL-1R domain-containing adaptor-inducing IFN- β

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

Introduction

Type I (IFN- α/β s) and type III IFNs (IFN- λ 1/IL-29, IFN- λ 2/IL-28A, IFN- λ 3/IL-28B) are conventionally regarded as mediators of antiviral immunity, but recent evidence suggests that type I IFNs also play an important role in host defense against bacterial pathogens [1]. The induction of type I IFNs is controlled mostly on a gene transcription level and has been studied in great detail. For activation of the IFN- β gene, IRFs, NF- κ B, AP-1, and high mobility group proteins are recruited to the promoter elements [2]. When studied in viral infection, the transcriptional regulation of type III IFN genes resembles the regulation of type I IFNs [3, 4].

The induction of IFNs can only occur after efficient recognition of a pathogen. DCs are well equipped for this task, as they express a wide range of PRRs, which detect conserved microbial motifs, termed PAMPs. PRRs that specialize in cytoplasmic surveillance of microbial PAMPs include the NLR family and RLRs. The most studied group of PRRs is the TLR family, whose members are expressed on the cell surface as well as intracellularly in endocytic compartments. Several TLRs participate in the detection of bacteria. TLR4 and TLR5 recognize LPS and flagellin, respectively. TLR2 associates with TLR1 and TLR6 and is more important in detecting components of Gram-positive bacteria, such as lipoteichoic acid, lipopeptides, and peptidoglycan. TLR9 is responsible for the recognition of unmethylated CpG motifs present in bacterial DNA [5]. Recently, TLR7 was discovered to sense RNA of bacterial origin [6].

TLR signaling proceeds along Toll/IL-1R domains of adaptor molecules. Among TLRs, TLR4 is unique and is able to initiate MyD88-dependent signaling, leading to proinflammatory cytokine production, and TRIF-dependent production of type I IFNs [7]. Kagan et al. [8] discovered recently that the TLR4 agonist LPS leads to sequential activation of TIRAP-MyD88 and TRAM-TRIF signaling pathways. The induction of TIRAP-MyD88 signaling is initiated from the plasma mem-

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brane, whereas the internalization of the TLR4 complex to early endosomes triggers the activation of TRAM-TRIF signaling [8]. Transcriptional systems activated downstream of TLR signaling include NF- κ B, IRFs, and MAPK-activated factors [5].

TLR signaling and phagocytosis of microbes are tightly linked events in DCs. These professional phagocytes internalize pathogens for antigen presentation to naïve T cells, and TLR molecules occupied in this process may affect the kinetics and outcome of phagosome maturation [9, 10]. Importantly, some intracellular pathogens exploit the endocytic pathway to enter subcellular compartments. For example, *Salmonella typhimurium* uses a type III secretion system to invade host cells in a process resembling macropinocytosis, resulting in the formation of a unique vesicle, a *Salmonella*-containing vacuole [11]. In contrast, invasion proteins of *Listeria monocytogenes* target host cell clathrin for entry, after which, this Gram-positive organism escapes the endocytic/lysosomal pathway to be released into the cytosol [11–13].

Induction of type I IFNs has been reported in different types of bacterial infections, such as those caused by intracellular pathogens *S. typhimurium*, *Shigella flexneri* [1], and *L. monocytogenes* [1, 14] and extracellular *Streptococcus pyogenes* (group A streptococcus) [15], as well as *Streptococcus agalactiae* (group B streptococcus) [16]. The unifying theme seems to be the activation of the TBK1/IRF3 axis, which in some cases, is activated independently of TLRs, nucleotide-binding oligomerization-1/2, and RLRs [14, 16]. Recently, bacterial DNA has been suggested to serve as a ligand for the cytosolic DNA-dependent activator of IRFs {double-stranded RNA activated inhibitor of translation (DAI) [17]} or other DNA-recognizing receptors and to trigger the induction of type I IFNs [18]. Moreover, TLR7 and IRF1 have been reported to mediate host-protective type I IFN production in response to bacterial RNA of phagosomal bacteria [6]. However, only few experiments have been conducted in primary human cells. Research has also focused on the induction of type I IFNs, whereas less attention has been paid to type III IFNs. Similar to type I IFNs, the type III IFN response can be activated by TLR4 and TLR9 ligands in DCs [19], but to our knowledge, there have been no reports investigating type III IFNs induced by live bacteria.

The primary focus of this work is on the regulation of types I and III IFNs in bacteria-infected human moDCs. We also report that the expression of IFNs is dependent on the uptake of *S. typhimurium*, which serves as a model organism for an intracellular Gram-negative bacterium.

MATERIALS AND METHODS

Cell culture

Leukocyte-rich buffy coats were obtained from healthy blood donors (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland). PBMCs were isolated by a density gradient centrifugation using Ficoll-Paque (GE Healthcare Bio-Sciences, Uppsala, Sweden), followed by isolation of monocytes by Percoll gradient centrifugation (GE Healthcare Bio-Sciences). Remaining T and B cells were depleted using anti-CD3 and anti-CD19 Dynal (Invitrogen, Carlsbad, CA, USA) magnetic beads. Monocytes (2.5×10^6 cells/well) were allowed to adhere to six-well plates (Falcon, Becton Dickinson, San Jose, CA, USA) for 1 h at 37°C in RPMI-1640 medium (Sigma-Aldrich, St. Louis,

MO, USA), supplemented with 0.6 μ g/ml penicillin, 60 μ g/ml streptomycin, 2 mM L-glutamine, and 20 mM HEPES. Nonadherent cells were removed by washing with cold PBS. Immature moDCs were generated by cultivating cells in complete RPMI medium in the presence of 10% FCS (Integro, BV, Dieren, The Netherlands), rhGM-CSF (10 ng/ml), and rhIL-4 (20 ng/ml; R&D Systems, Minneapolis, MN, USA), as described previously [20]. Fresh media were added every 2 days, and experiments were started at 6–7 days of cultivation.

Infections

Salmonella enterica serovar *typhimurium* ATCC 14028 strain was grown as described previously [20]. *Streptococcus thermophilus* was obtained from Valio Research Center (Helsinki, Finland) and cultivated as described [21]. MOI 5 was used throughout the study, except when assessing the bacterial uptake or phosphorylation of IRF3, where MOI 1 or MOI 10 was used, respectively (see below). Heat inactivation of bacteria was performed in 95°C dry heat block for 5 min. Sendai virus was used at MOI 5 [22].

Reagents

S. typhimurium ATCC 14028 smooth LPS (1 μ g/ml) preparation was from Sigma-Aldrich. CHX (Sigma-Aldrich) was used at 30 μ g/ml and added to the cells 30 min after infection. Cell signaling inhibitors to block signaling pathways used were PD98059 (specific MEK1 inhibitor, used at 10 μ M) and LY294002 (PI3K inhibitor, 50 μ M) from Calbiochem (Darmstadt, Germany) and SB202190 (specific p38 inhibitor, 10 μ M), SP600125 (specific stress-activated protein kinase/JNK inhibitor, 10 μ M), and PDTC (specific NF- κ B inhibitor, 100 μ M) from Alexis Biochemicals (Lausen, Switzerland). Inhibitors were added to the cells 30 min prior to infection. Dynasore was purchased from Sigma-Aldrich and used at 80 μ M, unless indicated otherwise. Cells were pretreated with dynasore for 10 min prior to infection, and for 24-h incubations, dynasore was added at a 9-h time-point of whole infection time.

ELISA

Secreted levels of CXCL10, TNF- α , and IL-6 were analyzed from cell culture supernatants using antibody pairs from BD PharMingen (San Diego, CA, USA). IFN- α 1 and IFN- α 2/3 were measured with a DuoSet ELISA kit (R&D Systems). PBL Biomedical Laboratories (Piscataway, NJ, USA) supplied VeriKine ELISAs to detect hIFN- β and hIFN- α (multi-subtype).

Quantitative RT-PCR

Cells were lysed with Trizol (Invitrogen). Total cellular RNA was isolated using the RNeasy mini kit (Qiagen, Crawley, UK). Cellular RNA (1 μ g) was reverse-transcribed into cDNA in TaqMan RT buffer with 5.5 mM MgCl₂, 500 μ M dNTPs, 2.5 μ M oligod(T)₁₆, 0.4 U/ μ l RNase inhibitor, and 1.25 U/ μ l MultiScribe RT (Applied Biosystems, Foster City, CA, USA). cDNA samples were then amplified in TaqMan Universal PCR master mix buffer (Applied Biosystems) using gene expression assay mix oligonucleotides (Applied Biosystems) to analyze mRNA levels for IFN- α 1 (Hs00601677_g1), IFN- α 2 (Hs00820125_g1), IFN- β (Hs00277188_s1), IFN- α 1 (Hs00256882_s1), CXCL10 (Hs00171042_m1), IL-6 (Hs00174131_m1), TNF- α (Hs00174128_m1), and IRF3 (Hs00155574_m1). The primer-limited β -actin VIC[®] assay (Applied Biosystems) was used for normalization. Each cDNA sample was amplified in duplicate with MxPro 3005P (Stratagene, La Jolla, CA, USA). The relative amounts of mRNAs were calculated with the $\Delta\Delta C_t$ method.

Western blot analysis

Whole cell lysates or nuclear proteins isolated as described previously [23] were run on 10% or 8% SDS-PAGE (30 μ g/lane), respectively, using the Laemmli buffer system. Proteins were transferred onto Immobilon-P (Millipore, Bedford, MA, USA) membranes. Immunoblotting was performed using rabbit anti-human Mx α [24], anti-Sendai (detects viral HA-neuraminidase and nucleoprotein) [25], anti-human actin (sc-10731, Santa Cruz Bio-

technology, Santa Cruz, CA, USA), anti-human P-IRF3 (Ser396; Cell Signaling Technology, Boston, MA, USA), and anti-human-IRF3 [22] antibodies. HRP-conjugated goat anti-rabbit antibodies (DakoCytomation, Glostrup, Denmark) were used for secondary staining. Protein bands were visualized on Kodak BioMax (Eastman Kodak, Rochester, NY, USA) films using the ECL system (Amersham Biosciences, Piscataway, NJ, USA).

Flow cytometry

moDCs were collected and washed with cold PBS. The cells were fixed with 1% paraformaldehyde for 15 min at room temperature, washed twice with PBS, and suspended in PBS + 2% FCS. The expression of cell-surface molecules was analyzed using FITC-conjugated anti-HLA class II and PE-conjugated anti-CD86 (Caltag, Burlingame, CA, USA) antibodies. Respective mouse isotype controls were used to monitor unspecific staining. Samples were run with FACScan or FACSCanto II (Becton Dickinson) and analyzed with FACSdiva software.

Bacterial uptake

Two hours prior to infection, the culture medium was changed to complete RPMI 1640 (10% FCS) without antibiotics. Dynasore or DMSO was added 10 min before infection with bacteria. Gentamicin protection assay was carried out as described previously [20]. Briefly, after 1 h infection with *S. typhimurium* (MOI 1), extracellular bacteria were killed with gentamicin (100 µg/ml) during a 1-h incubation. The number of intracellular bacteria was determined by CFU counting on Luria plates.

Online Supplemental material

Supplemental Fig. 1, provided online, shows that IFN-λ1 gene expression levels vary between moDCs originating from different blood donors, as measured by quantitative RT-PCR and ELISA.

RESULTS

LPS or *Salmonella*-induced expression of type III IFNs

To investigate the pattern of type III IFN expression in response to TLR4 agonist LPS or live *Salmonella*, we performed quantitative RT-PCR analysis using specific gene expression assays for IFN-λ1, IFN-λ2/3, IFN-β, and IFN-α1 (Fig. 1A). We observed that stimulation of moDCs with LPS or whole bacteria led to a robust and transient up-regulation of IFN-β and IFN-λ1 genes, the expression levels reaching their peaks at 2–4 h after infection. Interestingly, IFN-λ1 was also secreted by moDCs, but the production level varied considerably from one donor to another (Fig. 1B). To determine the biological variation of IFN-λ1 gene expression at the mRNA level, we performed quantitative RT-PCR from moDCs obtained from six different blood donors (Supplemental Fig. 1A). The level of IFN-λ1 gene expression varied considerably upon *Salmonella*

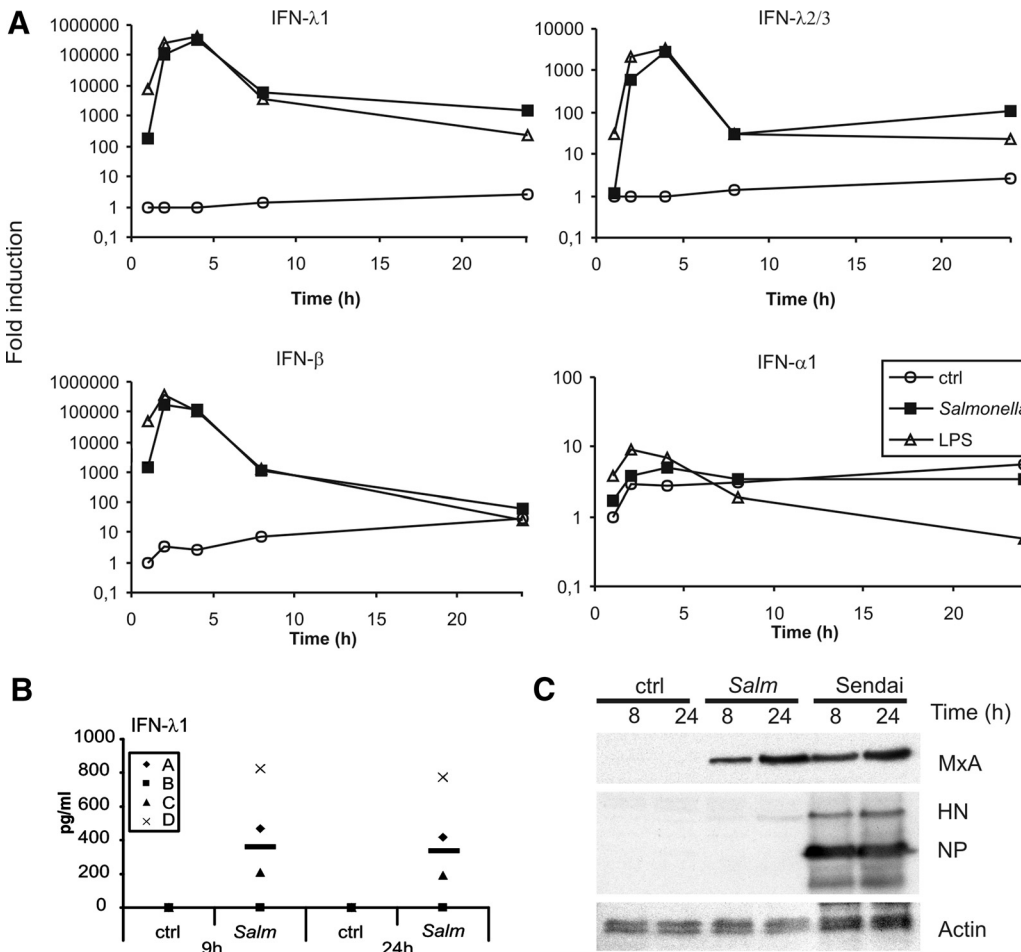


Figure 1. LPS and *Salmonella* trigger IFN gene expression in human moDCs. moDCs, obtained from four blood donors, were stimulated with LPS (1 µg/ml; A) or infected with *S. typhimurium* (*Salmonella*; MOI 5; A–C) or Sendai virus (MOI 5; C) for indicated times. (A) After stimulation, the cells were pooled for total cellular RNA isolation, and quantitative RT-PCR was performed to determine relative IFN-λ1, IFN-λ2/3, IFN-β, and IFN-α1 mRNA expression levels. The values were normalized against β-actin mRNA, and ΔΔCt results are shown as fold induction using control (ctrl) cell values at 1 h as a calibrator. (B) moDC supernatants of four blood donors were analyzed separately by ELISA for IFN-λ1 protein production. Thick lines represent the mean of IFN-λ1 levels. (C) moDCs from four different donors were pooled for obtaining whole cell extracts. Cellular MxA and Sendai virus proteins HA-neuraminidase (HN) and nucleoprotein (NP) were visualized by Western blotting. Equal input of proteins was confirmed by actin staining. All data are representative of three independent experiments.

stimulation; the donor with the highest gene expression showed as much as a 100-fold greater expression level of IFN- λ 1 mRNA than the donor with the least expression. In addition, mRNA expression data correlated well with ELISA results (Supplemental Fig. 1B). Considering that the trends of IFN- λ 1 gene expression were similar between all donors, in spite of great variation, we performed all of the other quantitative RT-PCR experiments using pooled moDCs, originally derived from four blood donors.

The expression of IFN- λ 2/3 mRNA was also clearly up-regulated, whereas the expression of IFN- α 1 mRNA was induced only weakly in response to stimulation (Fig. 1A). In contrast to IFN- λ 1, the production of IFN- λ 2/3 could not be detected at the protein level. Secreted IFN- α or IFN- β was detected in levels close to the detection limit of the ELISA kit (data not shown). Therefore, we performed Western blot analysis using anti-MxA antibodies to assess the ability of *Salmonella* to induce MxA protein expression as compared with Sendai virus, a robust inducer of IFN activity (Fig. 1C). The human MxA gene has two functional ISREs [26], and it is regulated specifically by type I and III IFNs [22, 27]. We noted that *Salmonella* triggered the production of MxA practically as efficiently as the Sendai virus.

Type III IFN gene expression triggered by *Salmonella* takes place in the absence of protein synthesis

To find out whether the synthesis of IFN- λ 1, IFN- λ 2/3, IFN- β , and IFN- α 1 mRNAs can be induced directly by bacteria, the cells were infected with *Salmonella* in the presence or absence of protein synthesis inhibitor CHX. Treatment of moDCs with CHX alone led to a weak increase (ca., 3-fold) in the steady-state levels of IFN- λ 1 and IFN- β mRNAs (Fig. 2). Apparently, ongoing protein synthesis was not required for IFN gene expression, as their expression was enhanced by *Salmonella* stimulation, also in the presence of CHX. In fact, stimulation of cells in the presence of CHX led to superinduction of all other IFN genes except that of IFN- λ 1. However, the expression of type I IFN-dependent gene CXCL10 [28] was blocked partially by CHX treatment. Importantly, CHX or *Salmonella* infection did not affect the mRNA level of IRF3, whose regulation is known to occur via post-transcriptional modifications [29].

Effects of MAPK, PI3-K, and NF- κ B inhibitors on *Salmonella*-induced type III IFN production in moDCs

The regulation of IFN- λ gene expression during viral infection has been reported to depend on the activation of IRFs and NF- κ B [3, 4]. To elucidate the mechanisms that participate in the induction of IFN- λ genes during bacterial infection, we tested selected pharmacological inhibitors of distinct signaling pathways. moDCs were infected with *Salmonella* in the presence or absence of inhibitors SB202190 (p38 MAPK inhibitor), PD98059 (MEK1 inhibitor), SP600125 (JNK inhibitor), Ly294002 (PI3K inhibitor), and PDTC (NF- κ B inhibitor). The expression of IFN- λ 1, IFN- λ 2/3, IFN- β , and IFN- α 1 was studied at the mRNA level 3 h after infection (Fig. 3A) or at the protein level 9 h after infection (Fig. 3B). CXCL10 and TNF- α were included in the analysis to verify the correct action of

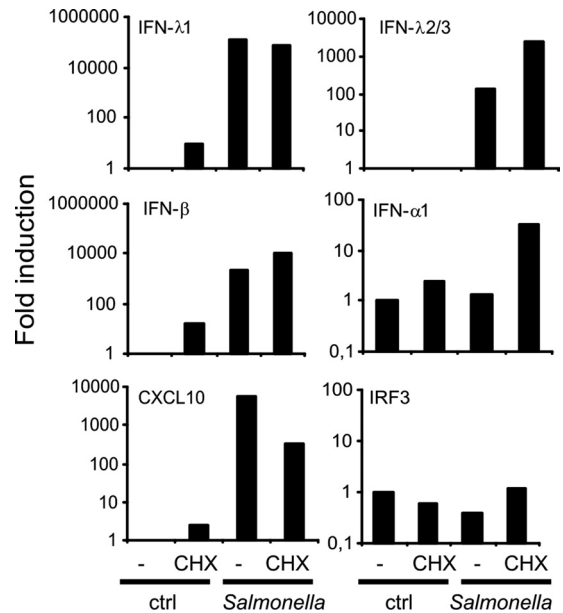


Figure 2. Effect of protein synthesis inhibition on *Salmonella*-induced IFN gene expression. moDCs from 4 blood donors were infected with *S. typhimurium* at MOI of 5, and after 30 min, protein synthesis inhibitor CHX was added at 30 μ g/ml. After 4 h infection, the cells were pooled, total cellular RNA was isolated, and quantitative RT-PCR was performed using IFN- λ 1, IFN- λ 2/3, IFN- β , IFN- α 1, IRF3, and CXCL10 gene-specific primers. The values were normalized against β -actin mRNA, and $\Delta\Delta$ Ct results are shown as fold induction using control cells (no CHX treatment) as a calibrator.

inhibitors. We observed that p38 MAPK and NF- κ B inhibitors similarly inhibited the up-regulation of IFN- λ 1 and IFN- β mRNAs. It is noteworthy that the production of IFN- λ 1 protein was sensitive to all other signaling inhibitors except the MEK1 inhibitor, which targets the ERK MAPK pathway. The induction of IFN- λ 2/3 mRNA, instead, was reduced by p38 MAPK, PI3K, and NF- κ B inhibitors. IFN- α 1 mRNA expression remained at a low level, but it was weakly enhanced by SB202190 and Ly294002 treatment. The induction of CXCL10 was impaired by SB202190, Ly294002, and PDTC at the mRNA and protein levels. TNF- α mRNA expression was not sensitive to the analyzed inhibitors, except for Ly294002, which caused a weak induction in TNF- α mRNA levels (Fig. 3A, bottom right). Secretion of TNF- α , however, was impaired by all three MAPK inhibitors.

Dynamin inhibitor dynasore impairs bacteria-induced moDC maturation and cytokine production

Up-regulation of CD86 and HLA class II molecules is a hallmark of DC maturation. It was reported recently that MyD88 and TRIF are required for maximal TLR4-induced murine DC maturation [30]. Moreover, Kagan et al. [8] demonstrated that TLR4 triggers TRAM-TRIF signaling from early endosomes, whereas TIRAP-MyD88 signaling is initiated at the plasma membrane. TRAM-TRIF-dependent signaling and subsequent production of IFN- β , IL-6, and CCL5 could be blocked by dynasore, a highly specific inhibitor of GTPase activity of dy-

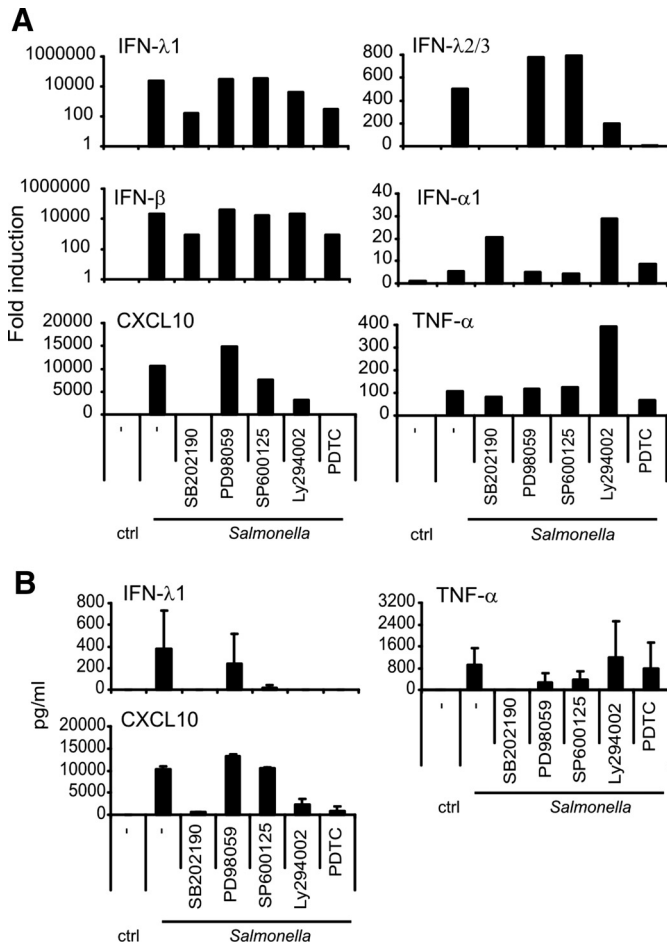


Figure 3. Cell signaling inhibitors interfere with *Salmonella*-induced IFN gene expression. moDCs from four blood donors were treated with inhibitors 30 min before infection with *S. typhimurium* (MOI 5). SB202190 (10 μ M), PD98059 (50 μ M), SP600125 (10 μ M), Ly294002 (50 μ M), and PDTC (100 μ M) were used to inhibit p38 MAPK, ERK MAPK, JNK MAPK, PI3K, and NF- κ B, respectively. (A) After 3 h infection, the cells were pooled, total cellular RNA was isolated, and quantitative RT-PCR was performed using IFN- λ 1, IFN- λ 2/3, IFN- β , IFN- α 1, CXCL10, and TNF- α gene-specific primers. The values were normalized against β -actin mRNA, and $\Delta\Delta$ Ct results are shown as fold induction using untreated control cells as a calibrator. (B) moDC supernatants were collected at 9 h after infection and analyzed by ELISA for IFN- λ 1, CXCL10, and TNF- α protein production. The mean \pm SD of four blood donors is shown. The results are representative of three independent experiments.

namin [31]. As the regulation of the IFN- λ 1 gene resembles that of IFN- β [3, 4], we tested whether dynasore has an effect on bacteria-induced moDC maturation and cytokine production.

First, we studied the expression of CD86 and HLA class II molecules in dynasore-pretreated, *Salmonella*-infected moDCs. We noted that the cell-surface levels of CD86 and HLA class II were down-regulated dose-dependently by dynasore treatment at 24 h (Fig. 4A). Similar results were obtained with TLR4 agonist LPS (data not shown). Dynasore-induced cell death was monitored by propidium iodide staining. When used at 80 μ M

concentration, cell viability was not affected (data not shown). Therefore, this concentration was chosen for further experiments. To investigate further the dependency of bacterial ligand uptake on moDC maturation and cytokine gene expression, we included another type of bacterium, Gram-positive *S. thermophilus*, in the experiments. *S. thermophilus* has been shown previously to induce efficient moDC maturation [21] and enhance the production of proinflammatory and Th1-type cytokines in human moDCs and PBMC [21, 32]. As shown in Fig. 4B, dynasore used at 80 μ M also decreased the expression of CD86 and HLA class II when cells were stimulated with *S. thermophilus*. This indicates that dynasore was able to block not only TLR4-LPS-dependent signaling but also signaling pathways triggered by Gram-positive bacteria.

Previously, LPS-induced CXCL10, IL-6, and IFN- β expression has been shown to depend entirely on TRAM-TRIF signaling [7, 8, 33, 34]. As the signaling pathways leading to the induction of IFN- λ 1 and IFN- β gene expression have been found to involve similar molecular mechanisms, we hypothesized that the induction of IFN- λ 1 in *S. typhimurium*-infected moDCs could be blocked by dynasore. We also measured CXCL10 and IL-6 mRNA expression in dynasore-treated moDCs infected with *S. typhimurium* or *S. thermophilus*. The expression of IFN-

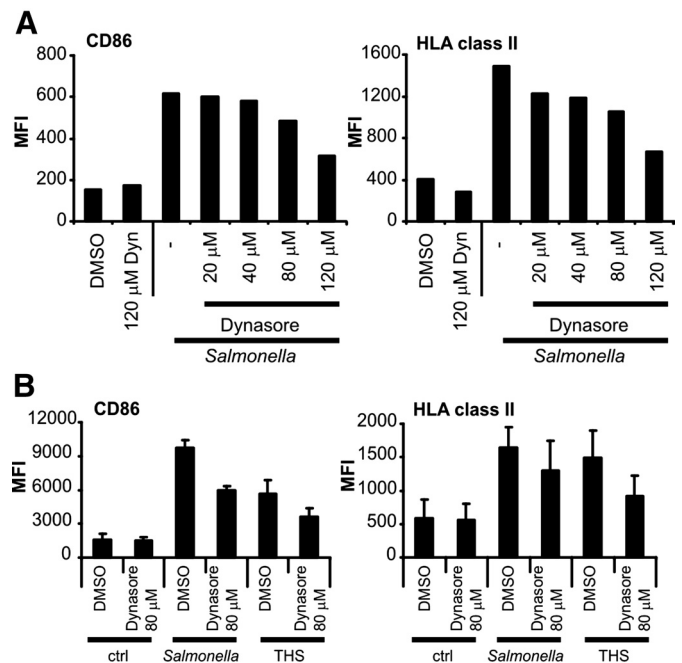


Figure 4. Dynasore impairs bacteria-induced moDC maturation. (A) moDCs were pretreated with DMSO or increasing amounts of dynasore, after which, cells were left uninfected or infected with *S. typhimurium* (MOI 5) for 24 h. The cells of four blood donors were pooled for analysis. (B) moDCs were pretreated with DMSO or dynasore (80 μ M), and then, cells were left uninfected or infected with *S. typhimurium* (MOI 5) or *S. thermophilus* (THS; MOI 5) for 24 h. The cells of three blood donors were analyzed separately. (A and B) The expression of CD86 and HLA II molecules was determined by flow cytometry. The data are shown as mean fluorescence intensities (MFIs) and are representative of two independent experiments.

λ 1, CXCL10, and IL-6 mRNAs was clearly reduced in *Salmonella*-infected moDCs that had been treated with dynasore (Fig. 5A). This was also the case in *S. thermophilus*-stimulated cells, except for IFN- λ 1 mRNA, which was not induced at 3 h and only weakly at later time-points (data not shown). At the same time, the expression of IRF3 mRNA remained unchanged. We also determined whether dynasore could impair the secretion of cytokines in bacteria-infected moDCs (Fig. 5B). The production of IFN- λ 1 was abolished completely by dynasore in *Salmonella*-infected moDCs. The production of CXCL10 and IL-6 was inhibited greatly by dynasore in cells infected with *S. typhimurium* or *S. thermophilus*.

These results suggest that dynasore impairs cytokine production by interfering with signaling pathways triggered by Gram-negative and -positive bacteria. To identify whether induction of IFN- λ 1 required viable bacteria, we stimulated moDCs with live or heat-inactivated *S. typhimurium* (Fig. 6). Live and heat-inactivated *S. typhimurium* could induce the production of IFN- λ 1. This production level was similar to that seen with LPS (1 μ g/ml) but considerably lower as compared with Sendai virus-infected cells. Bacteria-induced IFN- λ 1 levels were also low when compared with Sendai virus-infected cells. Similar to IFN- λ 1, heat inactivation of bacteria did not impair the production of CXCL10 or IL-6. In the presence of dynasore, the production of IFN- λ 1 was abolished in response to live or heat-inactivated bacteria, as well as LPS. Dynasore treatment did not, however, affect the production of IFN- λ 1 in response to the Sendai virus. The production of CXCL10 and IL-6 was impaired by dynasore treatment when cells were stimulated with bacteria, LPS, or Sendai virus.

Dynasore impairs the uptake of *Salmonella* in moDCs

The endocytosis of TLR4, as well as CD14 and LPS, has been shown to be dependent on dynamin-mediated processes [8, 35]. Moreover, the entry of uropathogenic *Escherichia coli*, *Yersinia pseudotuberculosis*, *Staphylococcus aureus*, and *L. monocytogenes* requires dynamin and clathrin in nonphagocytic cells

[12, 36]. As dynasore was able to inhibit *S. typhimurium*- and *S. thermophilus*-induced moDC maturation and cytokine production, we analyzed specifically whether bacterial uptake was inhibited by dynasore. The entry of *Salmonella* into moDCs was analyzed by a gentamicin protection assay [20]. moDCs were pretreated with dynasore, followed by infection with bacteria for 1 h. Extracellular bacteria were killed with gentamicin addition during the next 1-h incubation step, after which, host cells were lysed, and the number of intracellular bacteria was determined with viable count-plating. Dose-dependent inhibition of *Salmonella* uptake by dynasore was seen, and the 80- μ M concentration of the drug inhibited *Salmonella* entry significantly (~70% inhibition) into moDCs (Fig. 7A). Similar results were obtained in primary human macrophages (data not shown). However, full inhibition of bacterial entry was not seen, indicating that other coexistent or compensatory uptake mechanisms may be present.

Studies about mouse macrophages have shown that dynasore can inhibit the LPS-induced phosphorylation of IRF3, and NF- κ B and MAPK pathways remain unaffected [8]. Therefore, we wanted to analyze whether microbe-induced IRF3 phosphorylation is impaired by dynasore. We isolated nuclear proteins after 3 h infection with *S. typhimurium* or Sendai virus in the absence or presence of dynasore. As shown in Fig. 7B, the phosphorylation of IRF3 was clearly reduced by treatment with dynasore in response to *Salmonella* or Sendai virus infection.

DISCUSSION

The role of type I IFNs in bacterial infections has been highlighted in recent research [1]. However, the production and regulation of type III IFNs, which share functional similarities with type I IFNs, in bacterial infection have remained largely uncharacterized. In the present study, we have identified the signaling pathways and the role of dynamin-dependent endo-

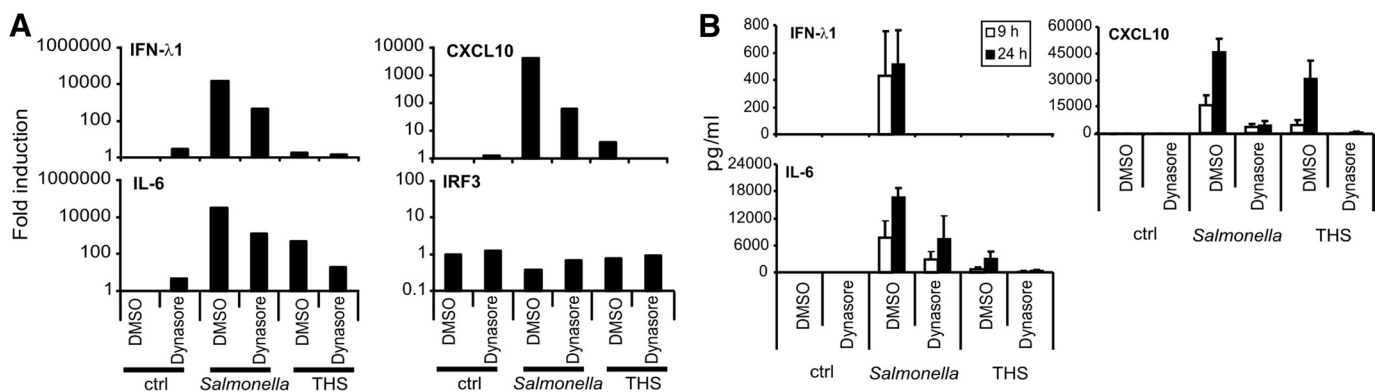


Figure 5. Dynasore impairs bacteria-induced cytokine production in moDCs. moDCs from four blood donors were pretreated with DMSO or dynasore (80 μ M), after which, cells were left uninfected or infected with *S. typhimurium* (MOI 5) or *S. thermophilus* (MOI 5). (A) The cells were collected 3 h after infection and pooled for isolation of total cellular RNA, and quantitative RT-PCR was performed to determine relative IFN- λ 1, CXCL10, IL-6, and IRF3 mRNA levels. The values were normalized against β -actin mRNA, and $\Delta\Delta$ Ct results are shown as fold induction using DMSO-treated, uninfected control cells as a calibrator. (B) Cell culture supernatants were collected at 9 or 24 h after infection, and the levels of IFN- λ 1, CXCL10, and IL-6 proteins were determined by ELISA. The mean \pm SD of four blood donors is shown. The results shown are representative of three independent experiments.

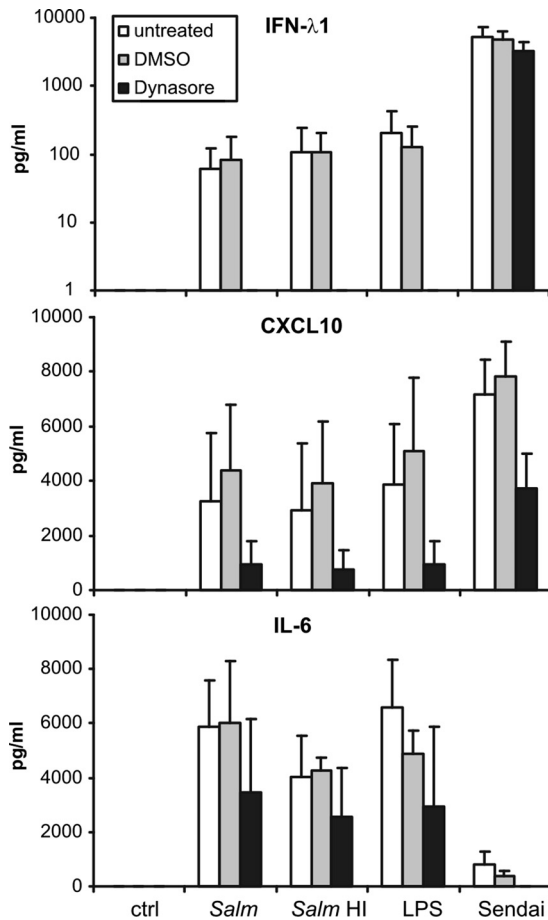


Figure 6. Live and heat-inactivated bacteria induce IFN-λ1 production equally. moDCs were pretreated with media, DMSO, or dynasore (80 μM), after which, cells were stimulated with live or heat-inactivated (HI) *S. typhimurium* (MOI 5) or LPS (1 μg/ml) or were infected with Sendai virus (MOI 5). Cell culture supernatants were collected at 9 h, and the levels of IFN-λ1, CXCL10, and IL-6 proteins were determined by ELISA. The mean ± SD of four blood donors is shown. Data are representative of two independent experiments.

cytosis in bacteria-induced types I and III IFN induction in human moDCs.

Our initial finding was that the mRNA expression of IFN-λ1 and IFN-λ2/3 was markedly increased in response to stimulation with TLR4 agonist LPS or infection with live *S. typhimurium* (Fig. 1A). The transient induction of the IFN-λ mRNAs represented a pattern similar to that of IFN-β, and the IFN-α1 subtype was only weakly up-regulated. We and others have previously reported similar results in viral infection and during TLR4 stimulation in human moDCs [4, 19]. Importantly, we also observed that IFN-λ1 was secreted by moDCs in response to live and heat-inactivated bacteria (Figs. 1B and 6), although this property varied from one donor to another. Furthermore, *S. typhimurium* infection triggered the induction of human MxA protein expression (Fig. 1C), which indicates indirectly that an efficient IFN response is elicited in moDCs infected with bacteria [22, 27]. The human type III IFN gene family consists of IFN-λ1, IFN-λ2, and IFN-λ3, out of which,

IFN-λ2 and IFN-λ3 are almost identical. In the mouse, however, IFN-λ1 is a pseudogene, and thus, IFN-λ2 and IFN-λ3 represent the functional subtypes of the type III IFNs [37]. This fundamental difference between mouse and man warrants further research of the type III IFN system in cells of human origin.

To understand the signaling requirements for induction of type III IFN genes versus type I IFN genes, we used a chemical inhibitor CHX to block de novo protein synthesis. It was clear that the mRNA induction was a direct consequence of *S. typhimurium* and moDC interaction, as blocking protein synthesis with CHX did not reduce the expression of IFN-λ1, IFN-λ2/3, IFN-β, or IFN-α1 mRNAs (Fig. 2). On the contrary, the expression of IFN-λ2/3 and IFN-β and IFN-α1 mRNA was up-regulated even further in the presence of CHX. Consistent with our observations in moDCs, CHX also enhanced IFN-λ1, IFN-β, and TNF-α mRNA expression in virus-infected primary

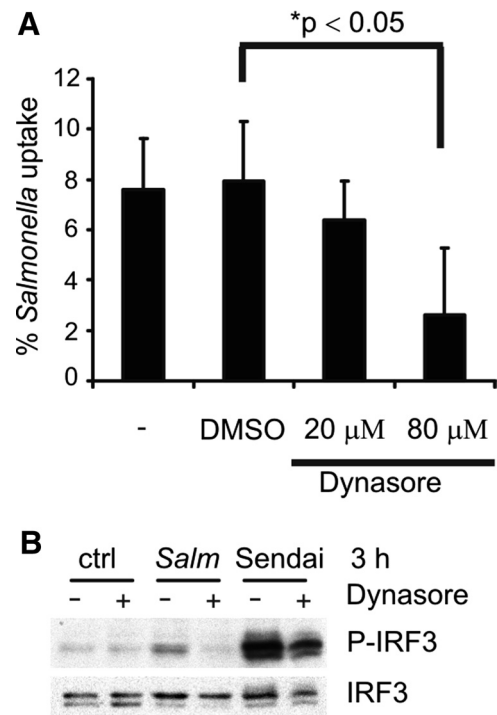


Figure 7. Dynasore inhibits bacterial entry and IRF3 phosphorylation. (A) moDCs were pretreated with media, DMSO, or dynasore (80 μM), followed by infection with *S. typhimurium* (MOI 1). After 1 h incubation, extracellularly remaining bacteria were killed with gentamicin (100 μg/ml). After 1 h incubation, host cells were lysed in the presence of 0.2% Triton X-100, and bacterial counts were determined by plating serial dilutions on Luria agar plates. Bacterial uptake is presented as the percentage of inoculated bacteria surviving gentamicin treatment. Results are the means (±SD) of three experiments performed with cells of four blood donors (n=12). *P < 0.05 between DMSO and dynasore 80 μM-pretreated, *S. typhimurium*-infected cells. (B) Nuclear protein extracts were prepared from moDCs after 3 h infection with *S. typhimurium* (MOI 10) or Sendai virus (MOI 5). Proteins were separated on 8% SDS-PAGE, and Western blotting was performed with antibodies detecting P-IRF3 or total IRF3 protein. Data are representative of two independent experiments.

human macrophages [38]. Taken together, the induction of IFN genes in *S. typhimurium* infection occurs without ongoing protein synthesis, and CHX probably stabilizes IFN mRNAs and/or blocks the action of negative-feedback mechanisms that could suppress the IFN response in bacterial infection.

The up-regulation of IFN- λ 1 and IFN- β mRNAs was similarly reduced by p38 MAPK and NF- κ B inhibitors (Fig. 3A), which indicates that these pathways positively contribute to the transcription of IFN genes in bacterial infection. In support of our findings, inhibition of p38 MAPK with SB203580 in virus-infected human macrophages has been shown to decrease the expression of IFN- β and IFN- λ 1 mRNAs [38]. At the protein level, the production of IFN- λ 1 was abolished completely by treatment with p38 and JNK MAPK, PI3K, and NF- κ B inhibitors (Fig. 3B), supporting the idea that multiple signaling pathways contribute to regulation of IFN- λ 1 gene expression. p38 MAPK, PI3K, and NF- κ B inhibitors also down-regulated the expression level of IFN- λ 2/3 mRNA, suggesting partially similar, regulatory mechanisms between IFN- λ 1 and IFN- λ 2/3 genes. However, inhibiting the action of ERK and JNK pathways seemed to display a weak stimulatory effect for the expression of IFN- λ 2/3, but this could not be confirmed at the protein level. The importance of the NF- κ B pathway in the regulation of IFN- λ 1 and IFN- λ 2/3 genes during viral infection has also been identified in previous studies. The promoter regions of IFN- λ 1 and IFN- λ 2/3 genes contain NF- κ B-binding sites, in addition to multiple IRF-binding ISREs and positive regulatory domain I sequences, and functional analyses have demonstrated that these sites are essential for IFN gene activation [3, 4].

PI3K is a multifaceted enzyme having important roles in various cellular processes, including a role in innate defense against microbial pathogens [39, 40]. Ly294002 prevents the activity of the PI3K pathway, and thus, we suggest that PI3K is one of the signaling components regulating IFN- λ synthesis in *Salmonella*-infected human moDCs. To our knowledge, PI3K has not been implicated previously in the regulation of IFN- λ genes. The role of PI3K in the regulation of IFN- β has been observed previously, although there is somewhat conflicting data about whether the role is positive or negative. Ly294002 has been reported to prevent IFN- β production in human plasmacytoid DCs or murine macrophages in response to TLR7 and TLR9 or TLR4 stimulation, respectively [41, 42]. In contrast, in another study, inhibition of PI3K in human mo-DCs by Ly294002 was found to enhance the synthesis of IFN- β during TLR3 and TLR4 stimulation [43]. In our experimental setting with *Salmonella*-stimulated human moDCs, Ly294002 conferred no effect on IFN- β mRNA expression, but it enhanced the induction of IFN- α 1 mRNA.

As a result of the observed resemblance in the regulation of IFN- λ 1 and IFN- β genes, we tested the effects of inhibition of dynamin-dependent endocytosis in moDCs with dynasore, which has been demonstrated to block LPS-induced expression of IFN- β , CCL5, and IL-6 [8]. First, we observed that pretreatment of moDCs with dynasore led to impaired expression of CD86 and HLA class II molecules in response to *S. typhimurium* and *S. thermophilus* (Fig. 4). The maturation of DCs and its dependence on MyD88 and TRIF signaling have been

studied mostly in mice. TLR4-mediated up-regulation of CD86 and MHC II has been reported to depend, at least partially, on TRIF but not on MyD88 [7, 30, 34, 44, 45]. Thus, we can assume that dynasore inhibits the up-regulation of CD86 and HLA II by blocking TRIF-dependent signaling, also in *S. typhimurium*-infected moDCs. The cell surface of Gram-positive *S. thermophilus*, in contrast, is rich in TLR2 agonists incapable of activating TRIF. As dynasore prevented the induction of TRAM-TRIF-dependent CXCL10 and IL-6 [7, 8, 33, 34] cytokines, also in response to *S. thermophilus* (Fig. 5), we propose that dynasore also interferes with TRIF- and LPS-independent signaling events in moDCs or that weak, previously unrecognized activation of TRIF pathways is taking place during stimulation of human moDCs with Gram-positive bacteria, such as streptococci.

Our results suggest that the mechanism by which dynasore prevented *S. typhimurium*-induced production of cytokines was probably related to the impaired phosphorylation of IRF3 (Fig. 7B). This observation is in line with the data from mouse macrophages, in which the phosphorylation of IRF3, but not the activation of NF- κ B or p38 MAPK, was inhibited by dynasore [8]. This suggests that *Salmonella*-induced phosphorylation of IRF3, which is triggered by internalization of bacteria, is a prerequisite for efficient IFN gene expression during bacterial infection. The entry of *Salmonella* into moDCs was clearly inhibited by dynasore treatment, which also led to inhibition of IRF3 phosphorylation (Fig. 7). This suggests that the internalization of *Salmonella* is a prerequisite for efficient IRF3 phosphorylation during the bacteria-host cell interaction process. Some inhibition of IRF3 phosphorylation was also found in dynasore-treated, Sendai virus-infected cells. However, in this case, the production of IFN- λ 1 remained unaffected (Figs. 6 and 7B). Even if the Sendai virus can fuse with the plasma membrane [46], some presently uncharacterized membrane events that are sensitive to dynasore-mediated inhibition may take part in the internalization process.

Moreover, the finding that dynasore prevented the internalization of *Salmonella* (Fig. 7) in moDCs provides evidence that dynamin-dependent endocytosis is important, not only in the endocytosis of LPS-TLR4 complex [8] but also in the uptake of whole live bacteria. Previously, dynasore has been shown to limit the invasion of type-1 pili-containing *E. coli* into bladder epithelial cells [36]. Veiga et al. [12] also described using nonphagocytic epithelial cells, where the entry of *Y. pseudotuberculosis*, *S. aureus*, and *L. monocytogenes* was inhibited by dynasore, and the invasion of *S. typhimurium* and *S. flexneri* remained unaffected [12]. The apparent discrepancy with some of the published data [12, 36] and ours may be explained by the cellular systems used, nonphagocytic versus phagocytic cells, respectively. In addition, although *S. typhimurium* is able to invade macrophages via *Salmonella* pathogenicity island 1-encoded virulence factors, *Salmonella* mutant strains impaired for cell entry are still actively taken up by DCs [47, 48]. Indeed, the use of dynasore, which inhibits the GTPase of host cell dynamin, strengthens the idea that DCs play an active role during *S. typhimurium* invasion.

S. thermophilus is a nonpathogenic, Gram-positive bacterium used in dairy products. We have shown previously that *S. ther-*

mophilus activates the production of proinflammatory cytokines and chemokines in human moDCs [21]. Among the prominently induced chemokines is CXCL10 (Fig. 5 and ref. [21]), whose production is thought to require the activation of NF- κ B and IRF3 [49, 50]. However, in contrast to TLR4 stimulation, TLR2 stimulation does not result in (detectable) IRF3 activation [49]. Therefore, we hypothesize that *S. thermophilus* is also internalized by human moDCs, and this event leads to the release of structural components of bacteria and thus, potential TLR, NLR, and RLR ligands from the phagosome. It is possible that the ligands triggering the production of CXCL10 are bacterial DNA or RNA, as it was discovered recently that in murine conventional DCs, the TLR7 pathway is activated by group B streptococcal RNA [6]. This study demonstrated further that IRF1 and IRF7, but not IRF3, were regulating IFN- β production in response to group B streptococci. The differential involvement of IRFs in bacterial infection is under intensive research, and we will pursue this line of research in human primary immune cells.

Evidently, the IFN response triggered by bacteria needs further investigation. As IFNs can confer beneficial or detrimental consequences to the host in bacterial infection [1], it is crucial to understand the signaling cascades and molecular events regulating bacteria-induced IFN responses in the human system.

AUTHORSHIP

T.E.P. designed the study, performed laboratory experiments, analyzed the results, and wrote the paper. S.L. performed laboratory experiments. P.Ö. designed the study and analyzed the results. I.J. designed the study and wrote the paper.

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