In Vivo Conditions Enable IFNAR-Independent Type I Interferon Production by Peritoneal CD11b⁺ Cells upon Thogoto Virus Infection

Georg Kochs,^a Martina Anzaghe,^b Stefanie Kronhart,^b Valentina Wagner,^a Patricia Gogesch,^b Stefanie Scheu,^c Stefan Lienenklaus,^d Zoe Waibler^b

Institute of Virology, Medical Center, Faculty of Medicine, University of Freiburg, Freiburg, Germany^a; Junior Research Group, Novel Vaccination Strategies and Early Immune Responses, Paul-Ehrlich-Institut, Langen, Germany^b; Institute of Medical Microbiology and Hospital Hygiene, University of Düsseldorf, Düsseldorf, Germany^c; Institute for Laboratory Animal Science, Hanover Medical School, and Institute for Experimental Infection Research, TWINCORE, Centre for Experimental and Clinical Infection Research, Hanover, Germany^d

ABSTRACT

Type I interferons (IFNs) crucially contribute to host survival upon viral infections. Robust expression of type I IFNs (IFN- α/β) and induction of an antiviral state critically depend on amplification of the IFN signal via the type I IFN receptor (IFNAR). A small amount of type I IFN produced early upon virus infection binds the IFNAR and activates a self-enhancing positive feedback loop, resulting in induction of large, protective amounts of IFN- α . Unexpectedly, we found robust, systemic IFN- α expression upon infection of IFNAR knockout mice with the orthomyxovirus Thogoto virus (THOV). The IFNAR-independent IFN- α production required *in vivo* conditions and was not achieved during *in vitro* infection. Using replication-incompetent THOV-derived virus-like particles, we demonstrate that IFNAR-independent type I IFN induction depends on viral polymerase activity but is largely independent of viral replication. To discover the cell type responsible for this effect, we used type I IFN reporter mice and identified CD11b⁺ F4/80⁺ myeloid cells within the peritoneal cavity of infected animals as the main source of IFNAR-independent type I IFN, corresponding to the particular tropism of THOV for this cell type.

IMPORTANCE

Type I IFNs are crucial for the survival of a host upon most viral infections, and, moreover, they shape subsequent adaptive immune responses. Production of protective amounts of type I IFN critically depends on the positive feedback amplification via the IFNAR. Unexpectedly, we observed robust IFNAR-independent type I IFN expression upon THOV infection and unraveled molecular mechanisms and determined the tissue and cell type involved. Our data indicate that the host can effectively use alternative pathways to induce type I IFN responses if the classical feedback amplification is not available. Understanding how type I IFN can be produced in large amounts independently of IFNAR-dependent enhancement will identify mechanisms which might contribute to novel therapeutic strategies to fight viral pathogens.

Type I interferons (IFNs) are a group of cytokines consisting of one single IFN- β , several IFN- α (14 functional isoforms in mice), and the less studied IFN- ϵ , - κ , - ω , - δ , - τ , and - ζ isoforms (1, 2). They are produced early after viral infections and critically contribute to host survival by constituting an early, overall antiviral state and initiating and shaping subsequent adaptive immune responses. Consequently, mice without a functional type I IFN system readily succumb to most viral infections (3, 4).

Production of type I IFNs is organized in two waves involving a positive feedback amplification loop (5). Pathogenic components such as viral nucleic acids, serving as pathogen-associated molecular patterns (PAMP), are sensed via specialized pattern recognition receptors (PRR). There are three main classes of PRR, consisting of the membrane-associated Toll-like receptors (TLR), located either at the outer plasma or the endosomal membrane, cytosolic RIG-I-like helicases (RLH; RIG-I and MDA-5), and NOD-like receptors (6–8). In addition, several cytosolic sensors for DNA have been described previously (9). Upon PAMP-ligation, PRR downstream signaling is initiated, resulting particularly in activation of the transcription factors NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) and IFN regulatory factor 3 (IRF3) (reviewed in references 10 and 11). This leads to the production of a minor, first wave of type I IFN, predominantly IFN- β and IFN- α 4 (5). This first wave is fairly small and

often cannot be detected systemically and thus is rather insufficient for full host protection.

However, these early type I IFNs bind in an auto- and paracrine manner to their receptor, the type I IFN receptor (IFNAR), which is expressed on almost all nucleated cells. IFN ligation initiates the so-called positive feedback amplification loop, inducing the second wave of type I IFN production. Upon IFNAR activation, proteins of the JAK/STAT (Janus kinase/signal transducers and activators of transcription) pathway are recruited to the receptor complex and get activated, resulting in formation of signaling complexes (12). These signaling complexes (particularly the IFN-stimulated gene factor 3) mediate IRF7 activation, which, in turn,

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Address correspondence to Zoe Waibler, zoe.waibler@pei.de.

G.K. and M.A. contributed equally to this article.

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promotes the production of late, large-scale type I IFN production, the second type I IFN wave (reviewed in references 13 to 15).

Collectively, it has been shown that robust type I IFN production both *in vitro* and *in vivo* critically depends on positive feedback amplification via the IFNAR (16–18). As a result, mice or isolated cells deficient for the IFNAR show substantially reduced type I IFN production upon infection (19).

Plasmacytoid dendritic cells (pDC), a rare and highly specialized subset of innate immune cells, are regarded as the main type I IFN-producing cell type. Even though *in vitro* virtually any cell type is able to produce type I IFNs in response to the appropriate stimulus, pDC were shown to produce up to 100 to 1,000 times more type I IFN than other cell types (reviewed in reference 20). Consequently, pDC were identified as being responsible for systemic type I IFN responses *in vivo* to a variety of infections (17, 20, 21). However, in mice depleted of pDC, considerable and protective type I levels can still be detected upon infection, indicating that, *in vivo*, other cell types are capable of initiating relevant type I IFN responses (22).

We previously showed that, in contrast to the situation in most other viral infections, in infections with Thogoto virus (THOV), myeloid dendritic cells (mDC) are the main type I IFN producers *in vitro* while pDC hardly secrete any type I IFNs (23). THOV is a segmented, single-strand, negative-sense RNA-encoded, tick-transmitted orthomyxovirus which is closely related to influenza virus (24). In contrast to studies of influenza virus isolates, THOV replication and pathogenesis can be studied optimally in laboratory mice (25). Interestingly, our previous studies with THOV demonstrated a robust type I IFN response upon *in vivo* infection of IFNAR-deficient mice, despite their inability to sense type I IFNs and to initiate the enhancing positive feedback loop via the IFNAR (23). Here, we unravel molecular mechanisms and determine the tissue and cell type capable of IFNAR-independent type I IFN expression upon THOV infection. Our data indicate that the host can effectively use alternative pathways to induce type I IFN responses if the classical feedback amplification via the IFNAR is not available. Understanding how type I IFNs can be produced by myeloid cells in large amounts independently of IFNAR-dependent enhancement might contribute to identifying mechanisms which could lead to novel therapeutic strategies to fight viral pathogens.

MATERIALS AND METHODS

Mice. All mice were bred under specific-pathogen-free (SPF) conditions at the Zentrale Tierhaltung of the Paul-Ehrlich-Institut. Wild-type (WT) C57BL/6 mice were purchased from Harlan. All knockout mice were backcrossed at least 10 times on the C57BL/6 background. Type I interferon receptor-deficient (IFNAR^{−/−}) mice have been described previously (3). MyD88-deficient (MyD88^{−/−}) mice were provided by Shizuo Akira (26), TRIF-deficient (TRIF^{−/−}) mice were provided by Bruce Beutler (27), and mitochondrial antiviral signaling protein (MAVS)-deficient (MAVS^{−/−}) mice were provided by Jürg Tschopp (28). Generation of MyD88/TRIF double-knockout mice was previously described (19). IFN-β^{Δβ-luc/Δβ-luc} knock-in reporter mice homozygously express firefly luciferase (FF-Luc) under the control of the endogenous IFN-β promoter (29). IFN-β^{mob/mob} knock-in reporter mice homozygously express an IFN-β-IRES-YFP (where IRES is internal ribosomal entry site and YFP is yellow fluorescent protein) bicistronic transcript under the control of the endogenous IFN-β promoter (30).

Mouse experimental work was carried out using 8- to 12-week-old mice in compliance with regulations of German animal welfare. For in-

fection, mice were anesthetized using isoflurane (CP-Pharma) and infected by the intraperitoneal (i.p.) route with a total volume of 200 μl of virus in phosphate-buffered saline (PBS). Animals were euthanized if severe symptoms were observed. To determine cytokine levels, peripheral blood was taken retro-orbitally upon anesthetization using isoflurane, and serum was prepared. IFN-α levels in serum were analyzed using an enzyme-linked immunosorbent assay (ELISA) kit (PBL Biomedical Laboratories).

Virus and virus-like particles (VLP). Thogoto virus (THOV) lacking the ML (where ML is an elongated form of the matrix protein) open reading frame was used in all experiments (31). THOV was propagated on BHK-21 cells and titrated on Vero cells. To analyze virus growth *in vivo*, organs were homogenized in medium using Lysing Matrix Tubes D (MP Biomedicals), and virus titers were determined by plaque assays. Vesicular stomatitis virus (VSV) Indiana, Mudd-Summers isolate, was propagated on BHK-21 cells and titrated on Vero cells.

For production of replication-incompetent virus-like particles (VLP), 293T cells were transfected with expression plasmids encoding the structural proteins of THOV and a reporter minigenome encoding enhanced green fluorescent protein (eGFP) flanked by the noncoding regions of THOV segment 5, as described previously (32). As a control, the expression plasmid encoding the viral matrix protein was omitted from the transfection mixture, resulting in a THOV-VLPΔM preparation that does not contain functional VLP. Cell culture supernatants were harvested at 48 h postinfection and subjected to ultracentrifugation for 2 h at 100,000 × g. The resulting pellets were resuspended in PBS, and a titer of 1 × 10⁷ fluorescence-forming units (FFU) per ml was determined by infecting Vero cells with 10-fold dilutions of the VLP preparations and counting eGFP-positive cells at 24 h postinfection.

Cell isolation and culture. Bone marrow (BM) cells were isolated by flushing femur and tibia of mice with RPMI medium supplemented with 10% fetal calf serum (FCS). Upon red blood cell lysis, cells were washed and seeded at a density of 1 × 10⁶ cells/ml or 2 × 10⁶ cells/ml in medium supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF; 100 ng/ml [R&D Systems]) or Flt3-L (100 ng/ml; R&D Systems), respectively. Flt3-L-supplemented cultures (pDC) were cultivated for 8 days with one medium change at day 4, whereas medium of the GM-CSF-supplemented cultures (mDC) was changed every 2 days, depending on the status of cultures, by replacing half of the medium with fresh cytokine-supplemented medium.

***In vitro* stimulation and quantification of cytokine production.** For stimulation, *in vitro*-differentiated DC were seeded at 1 × 10⁶ cells/well in 24-well culture plates in 1 ml of medium. CpG-containing oligodeoxynucleotide 2216 (CpG2216; ggGGGACGATCGTCggggg [uppercase letters, phosphodiester bond; lowercase letters, phosphorothioate bond]) (Sigma-ARK) was used at a concentration of 5 μg/ml. For UV irradiation of virus or VLP, a UV irradiation chamber (Herolab) was used. Irradiation with 75 mJ/cm² took approximately 10 s. After stimulation of cell cultures, cell-free supernatant was collected and analyzed using an ELISA kit to determine the amount of mouse IFN-α (PBL Biomedical Laboratories).

MACS. Mice were left untreated or were i.p. infected with 1 × 10⁶ PFU of THOV. At 6 h postinfection, spleens and peritoneal exudate cells (PEC) were isolated, and CD11b-positive cells were enriched using CD11b microbeads and an autoMACS Pro Separator (both from Miltenyi) according to the manufacturer's instructions. Purity of magnetically activated cell sorting (MACS)-enriched CD11b-positive cells usually ranged between 47 and 73%; CD11b-negative cell fractions did not show any CD11b⁺ cells when subjected to fluorescence-activated cell sorting (FACS) analyses.

FACS. For FACS analyses, cells were stained with the following fluorochrome-labeled monoclonal antibodies and isotype controls: anti-CD11c-allophycocyanin (APC), anti-CD69-phycoerythrin (PE)-Cy7, anti-B220-PE, rat IgG2b-APC isotype control, and hamster IgG1-APC isotype control (all from BD Pharmingen); anti-CD11b-Pacific Blue and

rat IgG2b-Pacific Blue isotype control (both from Caltag/Invitrogen); anti-F4/80-APC, anti-F4/80-PE, and rat IgG2b-PE isotype control (all from AbD Serotec); and rat IgG2a-PE isotype control (from Biozol). All analyses were performed using a BD LSR II flow cytometer and BD FACSDiva software (BD Biosciences).

Luciferase assay. For measurement of luciferase activity, cells were lysed in passive lysis buffer (Promega). The lysates were centrifuged at $10,000 \times g$, and luciferase activity in the supernatants was determined using a luciferase assay system (Promega).

Quantitative RT-PCR. Cells were lysed in peqGOLD TriFast (PiqLab), RNA was isolated from the aqueous supernatant using an RNeasy minikit (Qiagen), and 10 ng of RNA was analyzed with a one-step QuantiTect SYBR green reverse transcription-PCR (RT-PCR) kit (Qiagen). Cellular RNA was quantified with QuantiTect primers specific for mouse IFN- β (for Mm_Ifnb1_1, primer QT00249662), IFN- α 2 (Mm_IFN α 2, QT00253092), RIG-I (Mm_Ddx58_1_SG, QT00123515), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; QT01658692). To detect THOV segment 5 transcripts, a strand-specific primer pair (GenBank accession number [NC_006507](#), positions 501 to 520 and 615 to 596) was used. Values were normalized against GAPDH using the $\Delta\Delta C_T$ (where C_T is threshold cycle) method (33).

RESULTS

IFNAR-independent type I IFN responses upon THOV infection. We previously observed that THOV induces robust IFN- α and IFN- β secretion in mice independently of IFNAR signaling (23). As shown in Fig. 1A (left panel), intraperitoneal (i.p.) infection of mice with THOV indeed induced systemic production of IFN- α within 24 h even in the absence of type I IFN signaling via the IFNAR. However, for most viral infections, including infection with the single-stranded RNA (ssRNA)-encoded vesicular stomatitis virus (VSV), robust type I IFN induction depends on positive feedback via the IFNAR (19, 34) (Fig. 1A, right panel). How THOV infection enables efficient type I IFN production independently of this positive feedback signaling is still unclear.

In order to get a first insight into the cell type(s) responsible for IFNAR-independent IFN- α production, we generated pDC and mDC from bone marrow and isolated peritoneal exudate cells (PEC), mainly myeloid cells and macrophages, from the peritoneal cavity (35; also data not shown) of WT and IFNAR-deficient mice. Upon THOV infection, mDC and PEC from WT animals showed strong IFN- α secretion (Fig. 1B). However, IFN- α production by infected pDC was only marginal although these cells responded properly to CpG2216 stimulation (Fig. 1B). Quantitative RT-PCR analyses revealed a more than 150-fold-stronger THOV-specific signal in infected mDC than in pDC (Fig. 1C). This indicates a tropism of THOV for mDC, which most probably accounts for the reduced responsiveness of pDC toward THOV infection. Importantly, in contrast to the IFNAR-independent IFN- α production under *in vivo* conditions, cells isolated from IFNAR-deficient mice did not respond to THOV infection (Fig. 1B) although infection levels in IFNAR-deficient DC and their WT counterparts were equal (Fig. 1C).

Next, we aimed at investigating whether *in vivo* infection might be necessary for IFNAR-independent IFN- α secretion. We infected WT and IFNAR-deficient mice with THOV, isolated spleen cells and PEC at 6, 12, and 24 h postinfection, and cultivated the cells for an additional 24 h. PEC from infected WT animals secreted large amounts of IFN- α early after THOV infection (Fig. 2A). However, only minimal IFN- α could be detected in supernatants of PEC isolated from IFNAR-deficient animals at any time point investigated. Spleen cells of either WT or IFNAR-deficient

FIG 1 IFNAR-independent IFN- α induction by THOV *in vivo* but not *in vitro*. (A) C57BL/6 and IFNAR-deficient mice were i.p. infected with 2×10^4 PFU of THOV (left panel) or intravenously infected with 2×10^6 PFU of VSV. At the indicated time points, blood was withdrawn, and serum IFN- α was measured using an ELISA. (B) pDC and mDC were differentiated from bone marrow of uninfected WT and IFNAR-deficient mice. PEC (peritoneal exudate cells) were prepared from the same animals. Cells were THOV infected (multiplicity of infection of 0.1); controls were stimulated with CpG2216 (5 μ g/ml) or left untreated. At 24 h posttreatment, cell-free supernatants were harvested, and IFN- α was measured using an ELISA ($n = 6$ to 14). (C) pDC and mDC were differentiated from bone marrow of C57BL/6 (WT) and IFNAR-deficient (IFNAR^{-/-}) mice. Cells were THOV infected (multiplicity of infection of 0.1); controls were left untreated (uninf.). At 24 h posttreatment, cells were harvested, and THOV-specific transcripts were quantified via quantitative RT-PCR ($n = 2$ to 3 for both cell types). Data are given as fold changes compared to values in uninfected WT mDC. Error bars indicate standard deviations. ns, not significant; **, $P \leq 0.01$; ***, $P \leq 0.001$ (unpaired two-tailed t test); bd, below detection limit.

animals did not produce IFN- α (Fig. 2A). Hence, only minimal IFNAR-independent IFN- α secretion can be detected in *ex vivo* cultures upon *in vivo* infection.

In order to analyze *in vivo* IFN- α production directly in certain organs, we infected WT and IFNAR-deficient animals with THOV for 6, 12, and 24 h and determined IFN- α production in lysates of spleen, liver, and lung and in the fluid within the peritoneal cavity (peritoneal exudate [PE]). Of note, we detected early (at 6 and 12 h postinfection) IFN- α secretion only in the peritoneal cavity of infected WT mice. However, robust IFN- α levels were detected in the peritoneal cavity and spleen of WT and IFNAR^{-/-} mice at 24 h postinfection (Fig. 2B). At this time point, some IFN- α was also detected in livers and lungs of IFNAR^{-/-} mice, possibly due to enhanced virus replication. Later time points of analysis are not feasible for IFNAR-deficient mice as they succumb to THOV infection around 30 h postinfection (23). Other tissues or organs,

FIG 2 *In vivo* conditions are necessary for IFNAR-independent IFN- α expression. (A) C57BL/6 (WT; $n = 6$ to 8) and IFNAR-deficient (IFNAR^{-/-}; $n = 6$ to 8) mice were i.p. infected with 2×10^4 PFU of THOV. At the indicated time points after infection, spleen cells and PEC were isolated and cultured *in vitro* for 24 h without any additional stimulus. Cell-free supernatants were harvested, and IFN- α was measured using an ELISA. (B) C57BL/6 (WT; $n = 4$ to 8) and IFNAR-deficient (IFNAR^{-/-}; $n = 4$ to 8) mice were i.p. infected with 2×10^4 PFU of THOV. At the indicated time points after infection, spleen, liver, lung, and cell-free peritoneal exudate (PE) were isolated. Organs were homogenized, and IFN- α in homogenates and the PE were measured using an ELISA. (C) C57BL/6 (WT; $n = 3$) and IFNAR-deficient (IFNAR^{-/-}; $n = 3$) mice were i.p. infected with 2×10^4 PFU of THOV. At the indicated time points after infection, PEC were isolated, pooled, and characterized by FACS analyses for F4/80, CD11b, and CD69 surface expression. In the upper rows, numbers indicate percentages of cells in the upper right quadrant (F4/80⁺ CD11b⁺ cells). In the lower rows, numbers indicate percentages of cells positive for CD69 (gated on F4/80⁺ CD11b⁺ cells). Error bars indicate standard deviations. ns, not significant (unpaired two-tailed *t* test).

such as mesenteric lymph nodes, brain, gut, and kidney, were negative for IFN- α (data not shown). To characterize cells within the peritoneal cavity, we isolated PEC at 6, 12, and 24 h postinfection of WT and IFNAR-deficient mice and investigated them by FACS analyses (Fig. 2C). As expected (35), most cells were doubly positive for the surface markers CD11b and F4/80, indicating a myeloid/macrophage phenotype. In particular, this double-positive cell fraction decreased during the course of infection. Of note,

remaining CD11b⁺ F4/80⁺ cells showed an activated phenotype upon infection, as indicated by CD69 upregulation. This activation was dramatically reduced in IFNAR-deficient mice (Fig. 2C), which is in line with previous data showing that CD69 upregulation is, at least in part, type I IFN dependent (36).

Collectively, these data suggest that 6 h of *in vivo* infection are sufficient to induce secretion of IFN- α within the next 24 h of *in vitro* culture. After 24 h of *in vivo* infection, cells are no longer able to produce IFN- α within the next 24 h in *in vitro* culture, most probably because they do not survive infection that long. This suggestion is supported by the FACS data showing that the CD11b⁺ F4/80⁺ cell population within the peritoneal cavity gets activated between 6 and 12 h postinfection and then fades. In line with this observation, no IFN- α can be detected at 6 h postinfection without subsequent *in vitro* culture, but IFN- α accumulates within the peritoneal cavity at 24 h postinfection.

Thus, THOV infection enables robust IFN- α production independently of the positive feedback loop via the IFNAR. However, this requires *in vivo* conditions which are not present during *in vitro* infection and *ex vivo* cultivation.

IFNAR-independent type I IFN production upon THOV-VLP infection does require viral polymerase activity but not viral replication. It is generally accepted that viral replication is enhanced in the absence of a functional type I IFN system. This holds true for THOV infections as well (23). In order to investigate the IFNAR dependence of IFN- α production in the absence of excessive THOV replication, we generated replication-incompetent but transcriptionally active THOV-derived virus-like particles (THOV-VLP) carrying a minigenome encoding eGFP.

Intraperitoneal infection of WT and IFNAR-deficient mice with 10^6 FFU of eGFP-encoding THOV-VLP induced systemic IFN- α secretion, indicating robust activity of the VLP-encapsidated nucleocapsids in the absence of viral replication (Fig. 3A). Accordingly, UV irradiation abolished the IFN- α -inducing capacity of THOV-VLP, indicating the necessity for THOV-VLP transcriptional activity (Fig. 3B). FACS analyses for eGFP-positive cells, a measure for viral polymerase activity, demonstrated high fluorescence signals in PEC isolated from THOV-VLP-infected WT and IFNAR-deficient animals (Fig. 3C), suggesting that cells in the peritoneal cavity contribute to IFN- α levels detected in the serum. Moreover, as observed for THOV (23), THOV-VLP infection was sensed via RLH (MAVS-dependent) but not via TLR (MyD88- and TRIF-dependent) to induce IFN- α secretion (Fig. 3D).

We additionally used THOV-VLP for *in vitro* infection of differentiated pDC and mDC and isolated PEC. As a control, cells were incubated with a preparation that lacked M, the viral matrix protein, resulting in a THOV-VLP Δ M preparation that does not contain functional VLP. As observed for THOV-infected cells (Fig. 1B), THOV-VLP infection but not the THOV-VLP Δ M preparation strongly induced IFN- α secretion by WT mDC and, to a lesser extent, by WT pDC and PEC (Fig. 3E). Accordingly, FACS analysis showed a clear tropism of THOV-VLP for mDC and PEC (Fig. 3F). However, cells from IFNAR-deficient animals were unresponsive to THOV-VLP treatment (Fig. 3E) even though they were infected comparably (Fig. 3F), again indicating a dominant role of myeloid cells in type I IFN production upon THOV infection and the need of *in vivo* conditions for IFNAR-independent IFN- α induction.

CD11b⁺ F4/80⁺ cells from the peritoneal cavity account for IFNAR-independent type I IFN production. In order to identify

FIG 3 IFNAR-independent IFN- α induction by THOV-VLP. (A) C57BL/6 (WT; $n = 4$) and IFNAR-deficient (IFNAR $^{-/-}$; $n = 4$) mice were i.p. infected with THOV-VLP (10^6 FFU). At the indicated time points after infection, blood was withdrawn, and serum IFN- α was measured using an ELISA. (B) THOV-VLP were either left untreated or were UV irradiated (UV) prior to infection (5×10^4 FFU) of mDC differentiated from C57BL/6 mice. Controls were left untreated. At 24 h postinfection, cell-free supernatants were harvested, and IFN- α was measured using an ELISA. (C) C57BL/6 (WT; $n = 5$) and IFNAR-deficient (IFNAR $^{-/-}$; $n = 4$) mice were i.p. infected with THOV-VLP encoding eGFP by the viral minigenome (10^6 FFU) or were left untreated as a control (uninf.). At 48 h postinfection, spleen cells and PEC were analyzed for percentages of eGFP-positive cells by FACS. (D) PEC were isolated from C57BL/6 (WT), MyD88 $^{-/-}$ TRIF $^{-/-}$ (TLR $^{-/-}$), and MAVS $^{-/-}$ (RLH $^{-/-}$) mice and infected with THOV (multiplicity of infection of 0.1) or THOV-VLP (10^6 FFU). Controls were left untreated. At 24 h postinfection, cell-free supernatants were harvested, and IFN- α was measured using an ELISA. (E) Differentiated pDC and mDC as well as PEC were infected with THOV-VLP in three different concentrations (10^3 , 10^4 , or 5×10^4 FFU, as indicated by triangles below the graph). Controls were left untreated (—) or incubated with comparable amounts of a VLP preparation generated in the absence of viral matrix protein (THOV-VLP Δ M) ($n = 3$ to 12). At 24 h postinfection, cell culture supernatants were harvested, and IFN- α was measured using an ELISA. (F) pDC and mDC were differentiated from bone marrow of C57BL/6 (WT) or IFNAR-deficient (IFNAR $^{-/-}$) mice; PEC were isolated. Cells were infected with THOV-VLP encoding eGFP by the viral minigenome (10^6 FFU; black lines). Controls were incubated with comparable amounts of a VLP preparation generated in the absence of viral matrix protein (THOV-VLP Δ M; shaded curve). At 24 h postinfection, cells were analyzed for percentages of eGFP-positive cells by FACS (percentages are indicated). Data shown are representative for cells differentiated or isolated from 2 to 3 animals. Error bars indicate standard deviations. ns, not significant; **, $P \leq 0.01$; ***, $P \leq 0.001$ (unpaired two-tailed t test); bd, below detection limit.

the cell type in spleen and the peritoneal cavity responsible for IFNAR-independent type I IFN production, we used a reporter mouse system. We previously showed that IFN- α and IFN- β are coregulated upon THOV infection (23), and in contrast to the

FIG 4 IFNAR-independent type I IFN production by CD11b $^{+}$ PEC. (A) IFN- $\beta^{\text{mob/mob}}$ and IFN- $\beta^{\text{mob/mob}}$ IFNAR $^{-/-}$ mice ($n = 3$ to 6 for both genotypes) were i.p. infected with 2×10^4 PFU of THOV or were left untreated as a control. At 18 h postinfection PEC were isolated, and YFP reporter gene expression was analyzed in CD11b $^{+}$ and CD11c $^{+}$ cells by FACS analyses. (B) IFN- $\beta^{\text{mob/mob}}$ and IFN- $\beta^{\text{mob/mob}}$ IFNAR $^{-/-}$ mice ($n = 5$ for both genotypes) were i.p. infected with 2×10^4 PFU of THOV. At 18 h postinfection PEC were isolated and pooled, and YFP $^{+}$ cells were characterized by FACS analyses for F4/80, CD11b, CD11c, B220, and CD69 surface expression. Percentages indicate CD11b $^{+}$ CD69 $^{+}$ cells. (C) IFN- $\beta^{\Delta\text{Luc}/\Delta\text{Luc}}$ and IFN- $\beta^{\Delta\text{Luc}/\Delta\text{Luc}}$ IFNAR $^{-/-}$ mice ($n = 4$ for both genotypes) were i.p. infected with 10^6 PFU of THOV or were left untreated as a control. At 6 h postinfection PEC were harvested and subjected to MACS using CD11b-specific microbeads (+). The negative cell fraction (—) and unsorted cells (Ø) served as controls. For cells from each fraction, firefly luciferase (FF-Luc) activity was measured. (D) C57BL/6 (WT) and IFNAR-deficient (IFNAR $^{-/-}$) mice ($n = 3$ for both genotypes) were i.p. infected with 1×10^6 PFU of THOV. At 6 h postinfection PEC were isolated, pooled, and subjected to MACS using CD11b-specific microbeads. THOV-, IFN- β -, IFN- α 2-, and RIG-I-specific transcripts were quantified via quantitative RT-PCR. Data are given as fold change compared to values for uninfected WT and IFNAR $^{-/-}$ CD11b $^{-}$ cells. Data shown are representative of two independent experiments. Error bars indicate standard deviations. ns, not significant; *, $P \leq 0.05$; **, $P \leq 0.01$ (unpaired two-tailed t test).

situation for IFN- α , well-established reporter mice for IFN- β expression are available. Hence, we used IFN- $\beta^{\text{mob/mob}}$ mice that express YFP along with IFN- β under the control of the endogenous IFN- β promoter to detect cells by FACS analyses (30). Upon THOV infection, we detected YFP-positive (YFP $^{+}$) cells in the peritoneal cavity when gating on CD11b $^{+}$ cells in both IFN- $\beta^{\text{mob/mob}}$ and IFN- $\beta^{\text{mob/mob}}$ IFNAR $^{-/-}$ mice (Fig. 4A). In con-

trast, with gating on CD11c⁺ cells, only a slight increase in YFP⁺ cells was detectable in IFN- $\beta^{\text{mob/mob}}$ mice upon THOV infection, but this was absent in IFN- $\beta^{\text{mob/mob}}$ IFNAR^{-/-} mice (Fig. 4A). YFP⁺ CD11b⁺ cells were also greatly positive for the surface marker F4/80, again indicating their myeloid/macrophage phenotype (Fig. 4B). In line with this, YFP⁺ CD11b⁺ F4/80⁺ cells were mostly negative for CD11c and B220. As already shown for WT and IFNAR-deficient mice (Fig. 2C), cells from WT reporter mice also showed strong expression of the activation marker CD69, which was less pronounced in cells from IFNAR-deficient reporter mice (Fig. 4B). Interestingly, within the spleen, no YFP⁺ cells could be detected upon gating on either CD11b⁺ or CD11c⁺ cells (data not shown).

In order to verify these data within a second reporter mouse system, we used the luciferase reporter mouse model. Here, the firefly luciferase is expressed under the control of the endogenous IFN- β promoter (29). We enriched CD11b⁺ cells from the peritoneal cavity of THOV-infected IFN- $\beta^{\Delta\beta\text{-luc}/\Delta\beta\text{-luc}}$ and IFN- $\beta^{\Delta\beta\text{-luc}/\Delta\beta\text{-luc}}$ IFNAR^{-/-} animals by the MACS technique. Analyzing luciferase activity in total PEC before MACS enrichment or in the CD11b-negative and CD11b-positive cell fractions demonstrated that, indeed, CD11b⁺ cells from both WT and IFNAR-deficient reporter mice showed enhanced IFN- β promoter activity upon virus infection (Fig. 4C). Again, CD11b⁺ cells isolated from spleens of the same mice did not show any IFN- β promoter activity (data not shown).

Finally, we verified these data within WT and IFNAR-deficient mice. We infected mice with THOV, separated CD11b⁺ from CD11b⁻ cells by the MACS technique, and analyzed cell fractions by quantitative RT-PCR. These data show that CD11b⁺ cells from both WT and IFNAR-deficient mice are more prone to THOV infection than their CD11b⁻ counterparts. Consequently, they show enhanced IFN- β and IFN- α 2 transcription. Of note, only CD11b⁺ IFNAR^{-/-} and not CD11b⁻ IFNAR^{-/-} cells showed type I IFN mRNA expression upon THOV infection. In line with IFNAR-dependent CD69 upregulation on CD11b⁺ cells (Fig. 2C and 4B), the type I IFN target gene RIG-I was upregulated upon THOV infection in both CD11b⁺ and CD11b⁻ cells of WT animals but not in cells from IFNAR-deficient mice (Fig. 4D).

Thus, these data suggest that upon THOV infection, mostly CD11b⁺ myeloid cells are infected, get activated, and produce type I IFNs IFNAR independently. In contrast, CD11b⁻ cells are less prone to THOV infection and are incapable of IFNAR-independent type I IFN production.

DISCUSSION

We demonstrated that in contrast to other viruses and artificial stimuli, THOV infection and treatment with replication-incompetent THOV-VLP lead to type I IFN responses even in the absence of positive feedback amplification via the IFNAR. This was rather unexpected since it is broadly believed that mice deficient for the IFNAR are unable to sense type I IFNs and, hence, to induce full-blown type I IFN production (19, 37). It has been shown before that some viruses or artificial stimuli, such as VSV and Newcastle disease virus (NDV) or artificial double-stranded RNA poly(I:C), can induce minor type I IFN responses in an IFNAR-deficient setting (38, 39). However, levels were dramatically reduced compared to those in their WT counterparts. In particular settings, pDC were shown to be the main type I IFN-producing cell type (38, 39), which is in line with the suggestion

that pDC might be capable of differential usage of the transcription factor IRF7 (40, 41). In contrast, we showed that myeloid cells such as bone marrow-derived mDC and CD11b⁺ F4/80⁺ cells from the peritoneal cavity, but not pDC, are most responsive upon THOV infection *in vitro* and *in vivo*, respectively (Fig. 1, 2, and 4) (23). Induction of type I IFN responses upon both THOV and THOV-VLP infection is managed primarily via the MAVS-adapted RLH rather than by MyD88/TRIF-adapted TLR (Fig. 3D) (23). These findings are supported by data showing that myeloid cells use RLH while pDC use the TLR pathway for pathogen detection (42). In accordance with this, we showed that both THOV and THOV-VLP show a tropism for mDC and PEC rather than pDC (Fig. 3F).

Performing a set of concerted *in vitro*, *ex vivo*, and *in vivo* experiments, we showed that while no cell type investigated produced IFN- α independently of IFNAR upon *in vitro* infection (Fig. 1B), minor levels of IFN- α could be detected when cells were isolated from THOV-infected IFNAR-deficient mice and cultured subsequently *in vitro* (Fig. 2A). In contrast, robust IFN- α production was observed in spleen and in the peritoneal cavity of IFNAR^{-/-} mice *in vivo* (Fig. 2B). These data point toward the need for *in vivo* conditions for robust IFNAR-independent type I IFN induction upon THOV infection. It was shown that, on an individual level, the infected cell is not necessarily the type I IFN-producing cell (43). However, our own preliminary experiments with mixed cultures of PEC and spleen cells and experiments using mixtures of infected and uninfected cells did not result in robust IFN- α secretion *in vitro* (data not shown). Whether *in vivo* conditions involve the interaction of several cell types and/or the cooperation between infected and uninfected cells will be a matter of future investigations.

Using replication-incompetent but transcriptionally active THOV-VLP, we demonstrated that IFNAR-independent type I IFN induction depends on viral polymerase activity but is largely independent of viral replication (Fig. 3A). Accordingly, UV irradiation abolished the IFN- α -inducing capacity of THOV-VLP, indicating the necessity for THOV-VLP transcriptional activity (Fig. 2B). Nevertheless, compared to IFN- α levels upon THOV *in vivo* infection, total IFN- α levels upon THOV-VLP *in vivo* infection were reduced (compare Fig. 1A and 3A). This suggests that the viral load is of great importance for the magnitude of IFN- α responses. For most viruses, including THOV, it has been shown that the viral load is much higher in an IFNAR-deficient experimental setting than in WT mice or cells (23). However, this higher viral load does not lead to enhanced type I IFN responses compared to those seen with the WT in any of these settings, e.g., using VSV (Fig. 1A) (34) or NDV (39), THOV and the Rift Valley fever bunyavirus (44) being the exceptions.

The cellular mechanism used by myeloid cells for robust IFNAR-independent IFN- α production might include enhanced basal expression of pathogen sensors, thereby enhancing the first, IFNAR-independent wave of type I IFN production. Such a mechanism was suggested by Hui et al. for infection with an H5N1 influenza virus, demonstrating that virus-induced mediators upregulated RIG-I in uninfected cells by paracrine effects contributing to amplified cytokine cascades (45). However, our own quantitative RT-PCR data do not suggest enhanced RIG-I expression in IFNAR-deficient cells (Fig. 4D). On the other hand, it was shown that type III IFNs can induce a type I IFN-like response in a restricted subset of cells (46) and might therefore prime IFNAR-

independent IFN- α production. However, our own preliminary results indicate that type III IFN signaling is not involved in IFNAR-independent IFN- α production upon THOV infection (data not shown). This is in line with data showing that in the mouse, type III IFNs are expressed in a tissue-dependent fashion and primarily act on epithelial cells *in vivo* (47). Finally, the absence of the induction of negative regulators via the IFNAR (12) may contribute to the strong IFNAR-independent IFN- α production in myeloid cells upon THOV infection as well. Why these mechanisms, which might account for the IFNAR-independent IFN- α secretion upon THOV infection, do not play a role in infection with other viruses that are unable to mount IFN- α responses in the absence of the IFNAR will be a matter for future investigations.

Using two different IFN- β reporter mouse systems, we demonstrated that CD11b⁺ cells from the peritoneal cavity, further characterized as being F4/80⁺ CD11c[−] B220[−], are the main cell type producing type I IFNs independent of the IFNAR. CD11b[−] cells and CD11c⁺ cells, respectively, from both WT and IFNAR-deficient animals were rather unresponsive upon infection (Fig. 4). These results are in line with data showing a clear tropism of THOV for myeloid CD11b⁺ cells and much less for pDC (Fig. 1C and 4D), which were shown to be CD11c⁺. However, in WT or IFNAR-deficient animals as well as in both reporter mouse systems used, we never detected any type I IFN or reporter gene signal, respectively, within the spleens (data not shown). Along this line, Bauer et al. recently showed that upon treatment of IFN- $\beta^{\text{mob/mob}}$ mice with CpG, a stimulus for pDC but not mDC or PEC to produce type I IFNs (Fig. 1B), only a minor and distinct population of pDC within the spleen was stimulated to express the YFP reporter gene (48).

Collectively, our data indicate that the infected host can effectively use alternative pathways to induce type I IFN responses if the classical feedback amplification is not available. Understanding how type I IFNs can be produced in large amounts in specialized cell types independently of the IFNAR-dependent enhancement will broaden our view of host strategies to fight viral pathogens.

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