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Concomitant TLR/RLH Signaling of Radiosensitive and Radiosensitive Cells Is Essential for Protection against Vesicular Stomatitis Virus Infection

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Several studies indicated that TLR as well as retinoic acid–inducible gene I–like helicase (RLH) signaling contribute to vesicular stomatitis virus (VSV)–mediated triggering of type I IFN (IFN-I) responses. Nevertheless, TLR-deficient MyD88−/−/C211 mice and RLH-deficient caspase activation and recruitment domain adaptor inducing IFN-β (Cardif)−/− mice showed only marginally enhanced susceptibility to lethal VSV i.v. infection. Therefore, we addressed whether concomitant TLR and RLH signaling, or some other additional mechanism, played a role. To this end, we generated MyD88−/−/Trif−/−/Cardif−/− (MyTrCa−/−) mice that succumbed to low-dose i.v. VSV infection with similar kinetics as IFN-I receptor–deficient mice. Three independent approaches (i.e., analysis of IFN-α/β serum levels, experiments with IFN-α/β reporter mice, and investigation of local IFN-stimulated gene induction) revealed that MyTrCa−/− mice did not mount IFN-I responses following VSV infection. Of note, treatment with rIFN-α protected the animals, qualifying MyTrCa−/− mice as a model to study the contribution of different immune cell subsets to the production of antiviral IFN-I. Upon adoptive transfer of wild-type plasmacytoid dendritic cells and subsequent VSV infection, MyTrCa−/− mice displayed significantly reduced viral loads in peripheral organs and showed prolonged survival. On the contrary, adoptive transfer of wild-type myeloid dendritic cells did not have such effects. Analysis of bone marrow chimeric mice revealed that TLR and RLH signaling of radiosensitive and radiosensitive cells was required for efficient protection. Thus, upon VSV infection, plasmacytoid dendritic cell–derived IFN-I primarily protects peripheral organs, whereas concomitant TLR and RLH signaling of radiosensitive stroma cells as well as of radiosensitive immune cells is needed to effectively protect against lethal disease.  The Journal of Immunology, 2014, 193: 3045–3054.

Host cells are alerted to virus infection by conserved pathogen components or by products of pathogen replication that may trigger pattern recognition receptors (PRR). PRR comprise transmembrane proteins such as TLR and C-type lectin receptors, cytosolic RNA detection systems, such as retinoic acid–inducible gene I–like helicase (RLH), DNA sensors including DAI, IFI16, cGAS, and AIM2, and presumably other not yet identified signaling platforms (1, 2). Previous studies indicated that viruses, such as the ssRNA-encoded vesicular stomatitis virus (VSV), are recognized by multiple mechanisms. Several TLRs including TLR4 (3), TLR3 (5), TLR7 (6), and recently identified TLR13 (7), LRRFIP1 (8), as well as RIG-I (9–13), have been implicated in VSV recognition. Additionally, protein kinase R is triggered by VSV RNA and seems to play a role in protection (14). Several studies showed that VSV stimulates plasmacytoid dendritic cells (pDC) in a TLR7-dependent manner, whereas myeloid DC (mDC), macrophages, and murine embryonic fibroblasts are activated by RIG-I stimulation. This suggested that TLR and RLH signaling contribute to antiviral responses in a cell type–specific manner (9, 10, 15), whereas the in vivo significance of these signaling platforms remained unclear.

In the case of VSV infection, the induction of protective type I IFN (IFN-I) is critically required to promote host survival. This was demonstrated by type I IFN receptor (IFNAR)–deficient mice that succumb to VSV challenge within 1 to 2 d, whereas wild-type (WT) mice control the virus without developing signs of disease (16). For VSV-mediated induction of IFN-I responses, TLR7 and RIG-I play a more dominant role than TLR3 (5, 6, 9). As shown in several different studies, upon viral infection, pDC can be one major producer of IFN-I (17–19). To study the significance of pDC in viral pathogenesis in greater detail, mice were generated that allowed selective in vivo depletion of pDC. Upon VSV infection of such pDC-depleted mice, at early time points, the induction of IFN-I responses was markedly reduced, whereas the

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Abbreviations used in this article: BM, bone marrow; Cardif, caspase activation and recruitment domain adaptor inducing IFN-β; C211, enhanced GFP; FlhL3, FlhL ligand; hps, h postinfection; IFN-I, type I IFN; IFNAR, type I IFN receptor; ISG, IFN-stimulated gene; LN, lymph node; mDC, myeloid dendritic cell; My, MyD88; MyTrCa−/−, MyD88−/−/Trif−/−/Cardif−/−; NF, nucleoprotein; OAS, 2′5′ oligo(guanosine)–2′5′ synthetase; pDC, plasmacytoid dendritic cell; PRR, pattern recognition receptor; qRT-PCR, quantitative real-time PCR; IFN-α, recombinant human IFN-α; rIFN-β, recombinant murine IFN-β; RIG-I, retinoic acid–inducible gene I, RLH, retinoic acid-inducible gene I–like helicase; STING, stimulator of IFN gene; Trif, Toll/IL-1R domain–containing adaptor inducing IFN-β; VSV, vesicular stomatitis virus; WT, wild-type.

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overall survival was normal (20). Thus, this model was not very informative about the pDC function in VSV pathogenesis. Interestingly, mice devoid of MyD88, which is an obligatory adaptor protein of all TLR except TLR3, the latter of which is linked to the adaptor Toll/IL-1R domain–containing adaptor inducing IFN-β (TIRI), showed only moderately enhanced sensitivity to i.v. VSV infection (21, 22). Mice carrying a nonfunctional caspase activation and recruitment domain adaptor inducing IFN-β (CARD; also known as mitochondrial antiviral signaling adaptor, virus-induced signaling adaptor, and IFN-β promoter stimulator-1), which is situated in the outer mitochondrial membrane and links activated RIG-I and MDA5 with downstream signaling molecules, were devoid of RLH signaling and showed only marginally impaired survival when compared with WT mice (23). Similar to Cardif-deficient mice, mice devoid of the intracellular DNA sensor stimulator of IFN genes (STING) also showed only slightly enhanced sensitivity to lethal VSV infection (24, 25). Because STING is predominantly an endoplasmic reticulum–resident protein linking RIG-I and DNA-mediated intracellular innate signaling, it was speculated that STING was required to exert effective RIG-I function (26). Collectively, these results support the hypothesis that upon VSV infection, TLR, RLH, and maybe other signaling platforms play a role in promoting protective IFN-I responses. Nevertheless, currently only very limited information is available about crosstalk between different PRR systems in responses. Nevertheless, currently only very limited information is available about crosstalk between different PRR systems in responses.

For VSV neutralization assay, blood samples were drawn at the indicated time points, centrifuged at 20,000 × g for 1.5 min, and serum was frozen at −20°C. For determination of IgG, sera were reduced with 280 mM 2-ME (Sigma-Aldrich) for 1 h at room temperature. For IgM analysis sera not treated with 2-ME were used. Sera were heat-inactivated at 56°C for 30 min. For IgG determination by ELISA methods following biotin labeling and streptavidin–HRP, sera were reduced with 280 mM 2-ME and incubated with 5% FCS and 2 mg/ml of the indicated reagents. For detection of IgM, sera were reduced with 280 mM 2-ME and incubated with 5% FCS and 1% Glutamax (Life Technologies) and incubated at 56°C for 30 min. Serial 2-fold dilutions were mixed with equal volumes of virus diluted to contain 3 × 10^3 PFU/ml. The serum–virus mixture was incubated for 1.5 h at 37°C and then transferred onto Vero cell monolayers grown in a 96-well plate. After 1 h of incubation at 37°C, the monolayers were overlaid with 100 μl MEM containing 1% methylcellulose and incubated for 24 h at 37°C. Then the overlay was removed, and the monolayer was fixed and stained using 0.5% crystal violet dissolved in 5% formaldehyde, 50% ethanol, and 4.25% NaCl.

For VSV plaque assay, tissues were homogenized in 1 ml MEM supplemented with 5% FCS (Sigma-Aldrich). Serial 10-fold dilutions of homogenates were transferred onto Vero cell monolayers in six-well plates and incubated for 1 h at 37°C. Monolayers were overlaid with 2 ml MEM containing 1% methylcellulose and incubated for 24 h at 37°C. Then the overlay was removed, and the monolayer was fixed and stained using crystal violet.

**Generation of BM-derived mDC and pDC and BM chimeric mice**

BM cells were isolated by flushing femur and tibia with RPMI 1640 medium supplemented with 10% FCS, 10 mM HEPES, 1 mM sodium pyruvate, 2 mM Glutamax, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Life Technologies), and 0.1 mM 2-ME. Cells were then cultured with RSC (growth factor) (Sigma-Aldrich) and washed with PBS. For in vitro differentiation of BM-mDC or BM-pDC, BM cells were seeded at a density of 1 × 10^6 or 2 × 10^6 cells/ml and incubated for 8 d in medium supplemented with 100 ng/ml GM-CSF (R&D Systems) or 100 ng/ml Flt3 ligand (Flt3-L; R&D Systems), respectively. pDC were purified using the Plasmacytoid Dendritic Cell Isolation Kit II (Miltenyi) following the manufacturer’s instructions. For generation of BM chimeric mice, mice were lethally irradiated with 9 Gy, and the following day, they were i.v. reconstituted with 1 × 10^7 BM cells of the indicated genotype. BM chimeric mice were used for experiments after at least 8 wk of recovery.

**Isolation of in vivo–differentiated pDC**

A total of 1 × 10^6 Flt3-L–producing B16 tumor cells was injected s.c. in one flank of WT mice. Tumor growth was monitored until a maximal diameter of 1.5 cm was reached (38, 39). Tumor-bearing mice containing between 10 and 15% Siglec-H^+ pDC in the spleen were sacrificed, and pDC were isolated from the spleen by negative MACS selection using the Plasmacytoid Dendritic Cell Isolation Kit II (Miltenyi Biotec).

**IFN-α/β determination by ELISA**

Serum was analyzed for IFN-α and IFN-β by ELISA methods following the manufacturer’s instructions (IFN-β ELISA: PBL Biomedical Laboratories; IFN-α ELISA: ebioscience). For determination of IFN-α, the incubation with the biotin–labeled anti–IFN-α Ab was performed for 1 h at room temperature followed by overnight incubation at 4°C.
Detection of bioluminescence and in vivo imaging

Mice were anesthetized using isoflurane. Immediately following i.v. injection of 100 μl luciferin (30 mg/ml in PBS)/20 g mouse weight, the mice were analyzed in an in vivo imaging instrument (IVIS Spectrum CT; PerkinElmer). The acquired images were analyzed using Living Image 4.3.1 software. For the detection of luciferase activity in different organs and lymph nodes, the respective organs were prepared at the indicated time points after adjuvant application and stored at −80°C until analysis. After tissue homogenization in Glyo Lysis Buffer (Promega), Bright Glo Luciferase Assay System (Promega) was used to determine bioluminescence activity using a plate reader (BioTek).

FACS analysis

For FACS analysis, 25 μl blood was stained with a VSV nucleoprotein (NP)–specific monoclonal (H-2Kk+ RGGYVQYLGD; ProImmune) for 10 min at room temperature. Then the samples were stained with anti-B220 Pacific Blue (BD Pharmingen), anti-CD3 Alexa Fluor 647 (CalTag Laboratories), anti-CD8 PE-Cy5 (Invitrogen), and anti-CD4 FITC (BioLegend) as indicated. Absolute cell numbers were determined by adding 25 μl AccusCheck Counting-Beads (Invitrogen) to the samples. Data were acquired on an LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Gene expression analysis

For quantification of mRNA expression, RNA was extracted from spleen and inguinal lymph nodes (LN) of mice or from 6 × 10^5 BM-mDC using NucleoSpin RNA Kit (Qiagen) following the manufacturer’s instructions. A total of 100 ng total RNA was used for cDNA synthesis using Primer-Script First Strand cDNA Synthesis Kit (TaKaRa) according to the manufacturer’s instructions, Primers and SYBR Green (Bioline) were added to ∼10 ng cDNA, and quantitative real-time PCR (qRT-PCR) was carried out. PCR reactions were run in a LightCycler 480 (Roche). Fold changes of target genes were normalized to housekeeping gene GAPDH (40). The following primers were used in this study: IFN-stimulated gene (ISG) 15 (5'-GAGGTAGGCCTGCAAGA-3', 5'-TTGCTCGGCACTGCTTCTCTT-3'), 25'-oligodinucleotide synthetase (25'-5' OAS: 5'-GGATGCCTG-GACGTATCTG-3', 5'-TCGCTGGCTAAGATG-3'), and GAPDH (5'-TGACCCACAACTGTTTAGC-3', 5'-GGCCATGGACTGTGGFCATGAG-3').

Treatment with rIFN and IFN-I–containing serum

BM-mDC were treated with serial dilutions of recombinant, Chinese hamster ovary cell–expressed, murine IFN-β (stock concentration: 179 μg/ml = 3.58 × 10^3 U/ml) for 2 h. Subsequently, cells were infected with VSV-GFP at a multiplicity of infection of 0.01. After 18 h incubation, cGEP expression of BM-mDC was monitored cytofluorometrically. For treatment with human IFN-α/β/Δ hybrid (IFN-α/β) (41), mice were anesthetized using isoflurane, and 1.6 μg (∼10^3 U) rIFN-α (stock concentration: 165 μg/ml = 1 × 10^3 U/ml reactive on mouse cells) in 50 μl PBS was administered s.c. into the neck.

To generate IFN-containing serum, C57BL/6 mice were infected with 2 × 10^6 PFU VSV-M2, and serum was collected 12 h postinfection (hpi) (42). The IFN-α content of the serum was analyzed by an ELISA method. In the described experiment, serum was used containing 2 ng/ml IFN-α.

Statistical analysis

Mean cytokine levels, viral and Ab titers, and cytotoxic lymphocyte responses were compared by the Mann–Whitney U test. For survival analysis, the Mantel–Cox survival analysis with log-rank statistics was used. A p value ≤0.05 was considered statistically significant. For statistical analysis, the software package GraphPad Prism Version 5.0 (GraphPad) was used.

Results

Concomitant TLR and RLH signaling is necessary to efficiently protect against i.v. VSV infection

To study the role of TLR and RLH signaling in the protection against VSV infection, TLR signaling-deficient MyTrΔ/− mice and CaΔ/− mice devoid of RLH signaling were i.v. challenged with 2 × 10^6 PFU VSV. As observed in previous studies, under such conditions, MyTrΔ/− and CaΔ/− mice showed enhanced sensitivity to VSV infection as indicated by ∼70% of MyTrΔ/− and 25% of CaΔ/− mice succumbing to infection within 15 d, whereas essentially all WT controls survived (Fig. 1A). At infection doses of 2 × 10^5 or 2 × 10^6 PFU, MyTrΔ/− and CaΔ/− mice controlled VSV as efficiently as WT mice (Fig. 1B, 1C). As readout for the induction of adaptive immunity, VSV-NP–specific CTL and VSV-neutralizing IgM and IgG responses were analyzed. Of note, previous studies showed that unlike VSV-neutralizing Ab responses, CTL did not critically contribute to the protection against lethal VSV infection (43–46). Upon i.v. challenge with 2 × 10^6 PFU VSV, WT controls, MyTrΔ/−, and CaΔ/− mice showed similar expansion of VSV-specific CTL peaking 1 wk postinfection at ∼20% VSV-NP–specific CTL among CD8+ T cells (Fig. 1D). The analysis of virus-neutralizing Ab responses revealed that similar to WT controls MyTrΔ/− and CaΔ/− mice mounted IgM responses on day 4 that switched to IgG on day 8 (Fig. 1E).

To next address whether redundant TLR and RLH signaling would suffice to protect against VSV infection or whether an additional mechanism was needed, we intercrossed MyTrΔ/− and CaΔ/− mice to obtain MyTrCaΔ/− mice devoid of TLR and RLH signaling. MyTrCaΔ/− mice were as sensitive to VSV infection as IFNARΔ/− mice (Fig. 1A, 1B), and even at low-dose infection with 2 × 10^5 PFU, MyTrCaΔ/− mice died within 2 d with similar kinetics as IFNARΔ/− mice (Fig. 1C). One day postinfection with 2 × 10^6 PFU VSV, neither in WT nor MyTrΔ/− mice were virus titers detectable in the periphery or in the CNS (Fig. 2A, 2B), whereas CaΔ/− mice displayed low viral burden in spleen and liver (Fig. 2A). In contrast, IFNARΔ/− and MyTrCaΔ/− mice showed elevated virus titers in all organs tested (Fig. 2A, 2B). These results demonstrated that in addition to TLR and RLH signaling, no other mechanism was needed to trigger protection against VSV infection.

TLR/RLH-ablated mice do not mount IFN-I responses upon VSV challenge

As in VSV-infected MyTrCaΔ/− mice survival kinetics and viral loads were reminiscent of IFNARΔ/− mice, we hypothesized that in the absence of TLR and RLH signaling, VSV infection would not trigger protective IFN-I responses. To study this, IFN-α and IFN-β serum protein levels were determined 24 h following i.v. infection of mice with 2 × 10^6 PFU VSV. Although WT, MyTrΔ/−, and CaΔ/− mice produced significant levels of IFN-α, in the serum of IFNARΔ/− mice reduced IFN-α and in MyTrCaΔ/− mice, no IFN-α was detected (Fig. 2C). Interestingly, no IFN-β was measurable in the serum of tested mice, except for IFNARΔ/− mice, which showed moderately enhanced IFN-β levels (Fig. 2D). To address whether minimal IFN-β induction played a role, we next studied the particularly sensitive IFN-β reporter mouse model expressing a luciferase gene under the control of the IFN-β promoter (IFN-βα/βΔ-luc) (33). These mice were bred on the MyTrΔ/−, CaΔ/−, or MyTrCaΔ/− backgrounds, and 24 and 48 h after i.v. infection with 2 × 10^6 PFU VSV, VSV in vivo imaging was pursued. Although IFN-βα/βΔ-luc IFN-βΔ-luc MyTrCaΔ/−, and IFN-βα/βΔ-luc CaΔ/− mice displayed an overall similar IFN-β induction, IFN-βα/βΔ-luc MyTrCaΔ/− mice showed no detectable luciferase expression (Fig. 3A, 3B). For measuring the luciferase activity directly in the organs of all genotypes, tissues were prepared following treatment with an enhanced infection dose of 2 × 10^6 PFU. The analysis revealed that IFN-βα/βΔ-luc and IFN-βα/βΔ-luc MyTrΔ/− mice showed similarly induced luciferase activity in spleen, cervical LN (Fig. 3C), inguinal LN (Supplemental Fig. 1A), and lumbar LN (Supplemental Fig. 1B), whereas IFN-βα/βΔ-luc CaΔ/− mice showed even slightly enhanced luciferase activity in these tissues (Fig. 3C, Supplemental Fig. 1A, 1B). Most importantly, also under conditions of enhanced virus infection dose, VSV-infected IFN-βα/βΔ-luc MyTrCaΔ/− mice did not show luciferase activity in the tested tissues (Fig. 3C, Supplemental Fig. 1A, 1B).
myeloid transdifferentiation (MyTr) models to study the role of TLR/RLH in IFN-I responses in mammalian cells. Collectively, we interpreted the absence of serum IFN-α such induction was detected (Fig. 3D, Supplemental Fig. 1C, 1D). To verify that TLR/RLH ablation did not affect IFNAR signaling, we next studied local IFNAR triggering by qRT-PCR analysis of ISG induction, and deficiency of ISG stimulation that upon VSV infection with 10^6 PFU. Although spleen and LN of WT, MyTrCa^-/-, and Ca^-/- mice showed induction of ISG 15 and 2',5' OAS, in MyTrCa^-/- mice as well as in IFNAR^-/- mice, no such induction was detected (Fig. 3D, Supplemental Fig. 1C, 1D). Collectively, we interpreted the absence of serum IFN-α, lack of IFN-β induction, and deficiency of ISG stimulation that upon VSV challenge of MyTrCa^-/- mice, no IFN-I responses were induced.

TLR/RLH-ablated mice retain normal IFN-I sensitivity

To verify that TLR/RLH ablation did not affect IFNAR signaling, BM-mDC generated from WT, IFNAR^-/-, MyTr^-/-, Ca^-/-, and MyTrCa^-/- mice were treated with recombinant murine IFN-β (rIFN-β), and the induction of ISG was determined. Except for IFNAR^-/- BM-mDC, BM-mDC of the other genotypes tested showed similar ISG 15 mRNA induction (Fig. 4A). To address whether a comparable ISG induction also conferred an equivalent antiviral state, BM-mDC generated from WT, MyTr^-/-, Ca^-/-, and MyTrCa^-/- mice were treated with serially diluted rIFN-β, then infected with VSV-eGFP, and following incubation for 18 h, GFP expression of infected cells was measured by FACS analysis. Although IFNAR^-/- BM-mDC did not show antiviral effects, following incubation with ≥4 U concentrations of rIFN-β, cells from all other genotypes tested were protected against VSV infection (Fig. 4B). These results indicated that MyTrCa^-/- BM-mDC retained a similar sensitivity for IFNAR triggering as WT BM-mDC.

To next address the IFNAR responsiveness of MyTrCa^-/- mice in vivo, WT controls and MyTrCa^-/- mice were i.v. challenged with 2 × 10^6 PFU VSV, and 4 and 8 h later, the animals were s.c. treated with 50 μl serum that was collected from VSV-M2–challenged WT mice and contained ~2 ng IFN-I. Serum treatment prolonged survival by 1 d when compared with VSV-infected MyTrCa^-/- mice treated with PBS (Fig. 4C). Because previous studies showed that recombinant human IFN-α/B (rIFN-α) exhibited strong reactivity on mouse cells (41), we next s.c. applied 1 × 10^5 U of rIFN-α 4 and 8 h postinfection. Interestingly,

**FIGURE 2.** VSV-infected MyTrCa^-/- mice do not produce serum IFN-I and show enhanced virus titers in tissues. WT, IFNAR^-/-, MyTr^-/-, Ca^-/-, and MyTrCa^-/- mice were i.v. infected with 2 × 10^6 PFU VSV, and 24 h later, virus titers were determined in spleen, liver, and lung (A) or in olfactory bulb, cerebrum, cerebellum, brain stem, and spinal cord (B) (n ≥ 3). Blood samples were drawn to determine serum concentrations of IFN-α (n ≥ 7) (C) and IFN-β (n ≥ 5) (D) by ELISA methods. All data presented are pooled from two to three independently performed experiments, or one representative experiment out of three similar ones is shown. Horizontal bars indicate means ± SEM. *p ≤ 0.05, ***p < 0.0001 one-tailed Mann–Whitney U test.
under such conditions, MyTrCa\(^{-/-}\) mice were fully protected (Fig. 4C).

Besides the fact that these experiments verified that not only MyTrCa\(^{-/-}\) cells but also MyTrCa\(^{-/-}\) mice retained IFN-I sensitivity, it also showed that high-dose treatment with rIFN-α conferred protection against VSV infection. These characteristics qualified MyTrCa\(^{-/-}\) mice as a model for adoptive transfer studies to test the contribution of selected immune cell subsets to promote protection against lethal VSV infection.

Adoptively transferred pDC, but not mDC, mount IFN-I responses that confer antiviral protection of peripheral organs

To study whether DC subsets contribute to protection against lethal VSV infection, BM cells isolated from WT mice were differentiated in medium supplemented with either Flt3-L or GM-CSF to obtain cultures that contained ∼30% Siglec-H\(^+\)B220\(^+\) pDC or ∼96% CD11c\(^+\) mDC, respectively. Upon adoptive transfer of 1 × 10\(^7\) Siglec-H\(^+\) pDC purified from WT BM-pDC cultures and subsequent VSV challenge, MyTrCa\(^{-/-}\) mice showed improved survival and died 4–6 d later than controls that did not receive pDC (Fig. 5A). On the contrary, adoptive transfer of BM-mDC cultures was not effective (Fig. 5A). In addition, MyTrCa\(^{-/-}\) mice carrying adoptively transferred pDC mounted increased neutralizing Ab responses when compared with pDC-treated WT mice (Fig. 5B). At the time of death, VSV-infected MyTrCa\(^{-/-}\) mice carrying WT pDC showed no or very low viral titers in peripheral organs, whereas high viral loads were detected within the CNS (Fig. 5C). In contrast, MyTrCa\(^{-/-}\) mice treated with BM-mDC showed high virus burden in all tissues tested (Fig. 5C).

To study the site where upon VSV infection of MyTrCa\(^{-/-}\) mice carrying adoptively transferred pDC IFN-I responses were induced, BM-pDC were generated from IFN-β\(^{-/-}\)-luc mice, and 1 × 10\(^7\) purified IFN-β\(^{-/-}\)-luc BM-pDC were adoptively transferred to MyTrCa\(^{-/-}\) mice. After VSV infection, such mice showed induction of luciferase activity at similar sites as in the studies with IFN-β\(^{-/-}\)-luc mice (compare Figs. 3A and 5D). No IFN-β induction was measured upon VSV infection of WT and MyTrCa\(^{-/-}\) mice adoptively transferred with IFN-β\(^{-/-}\)-luc BM-derived mDC (Fig. 5D). These results indicated that in MyTrCa\(^{-/-}\) mice carrying TLR/RLH-competent pDC VSV infection triggered IFN-β induction at similar sites as observed in TLR/RLH-competent animals, whereas mDC did not.

To quantify the number of pDC needed to confer protective effects, 1 × 10\(^7\), 1 × 10\(^6\), or 1 × 10\(^5\) pDC purified from WT BM-
To address whether also other immune cells or some resident tissue cell subsets were involved in mounting protective IFN-I responses, BM chimeric mice were generated in which either radiosensitive immune cells (MyTrCa-/−) or radiosistant stroma cells (WT > MyTrCa-/−) were refractory to VSV-mediated induction of IFN-I responses. Upon VSV challenge, MyTrCa-/− > WT and WT > MyTrCa-/− showed intermediate sensitivity, whereas WT > WT mice showed only minor lethality and MyTrCa-/− > MyTrCa-/− mice were as sensitive to infection as the original MyTrCa-/− mice (Fig. 7A). WT > WT controls, MyTrCa-/− > WT and WT > MyTrCa-/− mice showed a similar induction of VSV-specific CTL responses after 6 d (Fig. 7B), as well as high IgM- and IgG-neutralizing Ab responses after 8 d of infection (Fig. 7C), indicating that adaptive immunity was normally induced. Moreover, MyTrCa-/− > WT mice showed even elevated CTL responses and enhanced IgG serum levels (Fig. 7B, 7C). Interestingly, adoptive transfer of WT pDC into MyTrCa-/− > WT mice did not significantly prolong survival upon VSV infection (data not shown). These experiments revealed that for complete protection against VSV infection, IFN-I responses of pDC and presumably other immune cells as well as of radiosistant stroma cells were needed.

**Discussion**

In this study, we addressed the cooperation of TLR and RLH signaling in the induction of VSV-triggered IFN-I responses. To this end, we intercrossed MyTrCa-/− and Ca-/− mice to obtain MyTrCa+/−xCa+/− mice. Such mice were as sensitive to lethal VSV infection as IFNAR+/− mice and did not mount protective IFN-I. This observation was surprising because MyTrCa+/−xCa+/− mice were as resistant to intermediate- and low-dose VSV infection as WT mice. These data indicated that concomitant TLR and RLH signaling was necessary for efficient protection against lethal VSV infection. This mouse model allowed addressing the biological role of different immune cell subsets in virus recognition and their capacity in mounting protective IFN-I responses. Transferred pDC significantly prolonged survival of VSV infection and induced an antiviral state in peripheral organs, whereas virus still entered the CNS and finally killed the host. BM chimeric mice in which either radiosensitive immune cells or radiosistant stroma cells were TLR/RLH deficient showed an intermediate sensitivity to lethal VSV infection. Collectively, these data showed that pDC alone conferred swift IFN-I responses which controlled virus replication in peripheral tissues, whereas full protection depended on IFN-I production also of radioresistant cells and presumably of other immune cells.

Although in serum of VSV-infected WT mice high levels of IFN-α were induced, in VSV-infected MyTrCa+/− mice, no IFN-α was detected. Interestingly, IFN-β was neither discovered in the serum of VSV-infected WT nor MyTrCa−/− mice, whereas IFNAR−/− mice mounted slightly enhanced IFN-β responses. The latter ob-

**FIGURE 4.** IFN-I treatment confers antiviral effects in MyTrCa−/− BM-mDC and protects VSV-infected MyTrCa−/− mice. (A) BM-mDC were generated from WT, IFNAR−/−, MyTr−/−, Ca+/−, and MyTrCa−/− mice and treated with 400 U murine rIFN-β. After the indicated time, cells were harvested, and RNA was purified and examined by qRT-PCR for the induction of ISG 15 (n = 3). (B) BM-mDC of the indicated genotypes were treated for 2 h with the specified concentrations of murine rIFN-β and then infected with VSV-eGFP at a multiplicity of infection of 0.01. After 18 h incubation, eGFP expression of BM-mDC was monitored by flow cytometry, n = 6. *p ≤ 0.05 one-tailed Mann-Whitney U test. The data shown are pooled from two independently performed experiments, or one representative experiment out of three similar ones is shown. Horizontal bars indicate means ± SEM. (C) WT controls (circles) and MyTrCa−/− mice (diamonds) were s.c. treated at the indicated time points with PBS (white), 2 ng IFN-containing serum (red), or 1 × 10^5 U of rIFN-α (red with cross) and i.v. infected with 2 × 10^6 PFU VSV. Survival was monitored daily.

pDC were adoptively transferred to MyTrCa−/− mice. Similar to the experiments above, upon i.v. infection with 2 × 10^6 PFU VSV, mice treated with 1 × 10^5 purified pDC showed prolonged survival of 3–5 d, whereas adoptive transfer of 1 × 10^5 pDC conferred 1–3 d prolonged survival, and adoptive transfer of 1 × 10^5 pDC was not effective (Fig. 6A). To address whether in vivo--differentially infected pDC similarly conferred protection, WT mice were s.c. treated with 1 × 10^6 Flt3-L–expressing B16 melanoma cells that promoted enhanced development of endogenous pDC (38, 39). After 10–14 d, mice developed tumors with a diameter of ~1.5 cm and carried between 10 and 15% Siglec-H^−/−B220^− pDC in the spleen compared with <0.1% pDC in untreated mice. Upon adoptive transfer of 1 × 10^5 of such ex vivo-isolated and purified pDC, VSV-infected MyTrCa−/− mice showed a similarly improved survival as observed in experiments with purified pDC derived from in vitro–differenitated BM-pDC cultures (Fig. 6B). To assure that adoptively transferred pDC would be triggered by VSV in a TLR7-dependent manner, pDC were generated from BM of TLR7−/− mice. Upon in vitro stimulation with CpG, such cells showed induction of CD86 and CD69, whereas upon treatment with R848 as a direct trigger of TLR7, no upregulation of activation markers was observed (Supplemental Fig. 2). Following adoptive transfer of 1 × 10^5 TLR7−/− or WT pDC purified from BM-pDC cultures, only WT pDC but not TLR7−/− pDC conferred prolonged survival of VSV-infected MyTrCa−/− mice (Fig. 6C). Collectively, these results indicated that upon VSV infection, pDC were triggered in a TLR7-dependent manner to mount rapid IFN-I responses in secondary lymphoid tissues that protected peripheral organs, but which did not suffice to control virus replication within the CNS.

**VSV induces IFN-I responses of radiosensitive immune cells and of radiosistant stroma cells that together confer full protection**
servation can be explained by continued PRR triggering in absence of IFNAR feedback or by the lack of IFN-β uptake, as previously suggested in the context of *Listeria* infection (47). Nevertheless, local IFN-β induction was found in cervical LN and spleen of VSV-infected IFN-β<sup>+/+</sup>-luc reporter mice, which was absent in IFN-β<sup>+/+</sup>-luc MyTrCa<sup>−/−</sup> animals. To study local IFNAR triggering, ISG induction within secondary lymphoid organs was examined. Whereas spleen, cervical LN, inguinal LN, and lumbar LN of VSV-infected WT mice showed significant ISG 15 and 2′,5′ OAS mRNA induction, no such effects were detected in VSV-infected MyTrCa<sup>−/−</sup> mice. Because IFN-β<sup>+/+</sup>-luc reporter mice and the induction of ISG were sensitive readouts for IFN-I responses, even when elicited only locally without IFN-I appearing in the serum, we concluded that MyTrCa<sup>−/−</sup> mice did not mount IFN-I responses upon VSV challenge. Thus, concomitant TLR and RLH triggering was indispensable to promote survival of VSV infection of mice, whereas other additional mechanisms did not seem to be critically involved.

To verify that in MyTrCa<sup>−/−</sup> mice IFN-I–mediated IFNAR signaling was not perturbed, we generated MyTrCa<sup>−/−</sup>/IFNAR<sup>−/−</sup> BM-mDC, treated them with rIFN-β, and determined the induction of ISG 15 and of antiviral effects. Indeed, in these assays, WT and MyTrCa<sup>−/−</sup> BM-mDC showed very similar antiviral effects, whereas ISG 15 induction was even elevated in MyTrCa<sup>−/−</sup> BM-mDC. These data indicated that IFN-β–mediated IFNAR signaling was not impaired by MyTrCa ablation. Various studies showed before that during activation of multiple PRR pathways, antagonism between single signaling pathways may shape the overall response (27, 48). Thus, it can be speculated that also basal TLR and RLH signaling modulated ISG mRNA, which would explain enhanced ISG 15 mRNA induction upon IFN-β treatment of TLR- and RLH-deficient cells. To study in vivo whether MyTrCa<sup>−/−</sup> mice retained IFN-I sensitivity, mice were treated with IFN-I–containing serum or recombinant human IFN-α, which in previous studies was shown to be active in the murine system (41). To recapitulate kinetics of endogenous IFN-I responses, treatment with IFN-I–containing serum or rIFN-α was initiated 4 hpi and repeated at 8 hpi. Although treatment with serum containing ∼2 ng IFN-α prolonged survival of VSV-infected MyTrCa<sup>−/−</sup> mice by 1 d, treatment with 1 × 10<sup>5</sup> U rIFN-α conferred quantitative protection of MyTrCa<sup>−/−</sup> mice. These results indicated that: 1) the short <i>t</i><sub>1/2</sub> of serum IFN-I was not sufficient to mediate significant protective effects; 2) comparably high-level IFN-I responses were needed to protect MyTrCa<sup>−/−</sup> mice; and 3) in MyTrCa<sup>−/−</sup> mice, IFNAR signaling was preserved in all relevant cell subsets. The observation that IFN-I sensitivity of MyTrCa<sup>−/−</sup> mice was retained qualified MyTrCa<sup>−/−</sup> mice as a suitable model to study the in vivo function of WT immune cell subsets, such as DC, in an environment that is completely blinded for the innate recognition of VSV infection.
pDC have been described as main IFN-I producers in many different virus infections (17–19). To study their role in a gain-of-function setting, WT pDC isolated from BM-pDC cultures were adoptively transferred to MyTrCa−/− mice. Upon VSV infection, pDC carrying MyTrCa−/− mice showed prolonged survival by 4–6 d, whereas transfer of WT BM-mDC did not have such effect. Notably, a similarly prolonged survival of VSV-infected MyTrCa−/− mice carrying pDC isolated from BM-pDC cultures and MyTrCa−/− mice carrying pDC directly isolated from mice was observed. Although the ex vivo–isolated pDC were derived from mice carrying an Flt3-L–expressing melanoma cells, and 1 × 10^5 of such pDC was adoptively transferred to MyTrCa−/− mice and WT controls (n ≥ 6). The following day, mice were infected with 2 × 10^4 PFU VSV, and survival was monitored. (C) Total of 1 × 10^5 purified BM-pDC generated from TLR7−/− mice (n ≥ 5) was adoptively transferred to WT and MyTrCa−/− mice. The following day, mice were infected with 2 × 10^4 PFU VSV, and survival was monitored. **p ≤ 0.0082, ***p < 0.0001 log-rank, Mantel–Cox test.

TLR/RLH signaling is essential to control VSV

FIGURE 6. Increasing numbers of adoptively transferred TLR7-competent pDC enhance protection of MyTrCa−/− mice. (A) Upon adoptive transfer of 1 × 10^7, 1 × 10^6, or 1 × 10^5 purified WT BM-pDC, MyTrCa−/− mice were challenged with 2 × 10^8 PFU VSV, and survival was monitored (n ≥ 5). (B) pDC were purified from spleen of WT mice treated with Flt3-L–expressing melanoma cells, and 1 × 10^5 of such pDC was adoptively transferred to MyTrCa−/− mice and WT controls (n ≥ 6). The following day, mice were infected with 2 × 10^4 PFU VSV, and survival was monitored. **p ≤ 0.0082, ***p < 0.0001 log-rank, Mantel–Cox test.
chimeric mice, in which either radiosensitive immune cells or radioresistant stroma cells were TLR/RLH ablated, revealed that both immune cells as well as radioresistant stroma were needed to confer efficient protection against VSV infection. Interestingly, adoptive transfer of WT pDC into chimeric mice, in which immune cells were TLR/RLH deficient, whereas radioresistant cells were TLR/RLH competent, did not show prolonged survival upon VSV infection, suggesting that in addition to pDC other immune cells significantly contributed to protective IFN-I responses.

Because our data clearly demonstrated that interaction between TLR and RLH signaling was critically needed to confer protection against VSV infection, the question arose of how mechanistically this interaction could be envisaged. As published previously, pDC seem to be preferentially triggered by endosomal TLR, whereas mDC and many other cell subsets are primarily triggered by cytosolic TLR to mount IFN-I responses (15). Thus, one explanation for redundant TLR and RLH triggering needed for protection against VSV infection is that depending on the involved cell subset, either TLR or RLH plays a critical role for the induction of protective IFN-I. This may not only apply for immune cell subsets but also for radioresistant cells. A recent report showed that upon TLR triggering of pDC RIG-I expression was upregulated independent of IFNAR stimulation (57). This example indicated another level of TLR and RLH interaction (i.e., that in a first phase, cells are TLR-dependently triggered to become sensitive for RLH triggering). Currently, it is difficult to estimate the in vivo relevance of this mechanism for protection against VSV. Nevertheless, it is certainly possible that in particular on the level of radioresistant stroma cells, this mechanism might be important.

In conclusion, we found that MyTrCa/−/− mice were not triggered upon VSV infection to mount protective IFN-I and that such mice succumbed to infection with similar kinetics as IFNAR−/− mice. These data indicated that besides TLR and RLH signaling, no other signaling platforms were critically involved in VSV recognition. Because MyTrCa/−/− mice retained IFNAR sensitivity, these mice served as an ideal model to more precisely dissect the role of protective IFN-I responses in viral pathogenesis. We found that rapid IFN-I responses mounted by pDC induced an antiviral state in peripheral organs, whereas IFN-I production of radioresistant immune cells and of radioresistant stroma was needed to fully protect mice from virus entering the CNS.

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Disclosures

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