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Type I IFN-Mediated Protection of Macrophages and Dendritic Cells Secures Control of Murine Coronavirus Infection[1]

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The swift production of type I IFNs is one of the fundamental aspects of innate immune responses against viruses. Plasmacytoid dendritic cell-derived type I IFNs are of prime importance for the initial control of highly cytopathic viruses such as the mouse hepatitis virus (MHV). The aim of this study was to determine the major target cell populations of this first wave of type I IFNs. Generation of bone marrow-chimeric mice expressing the type I IFN receptor (IFNAR) on either hemopoietic or non-bone marrow-derived cells revealed that the early control of MHV depended mainly on IFNAR expression on hemopoietic cells. To establish which cell population responds most efficiently to type I IFNs, mice conditionally deficient for the IFNAR on different leukocyte subsets were infected with MHV. This genetic analysis revealed that IFNAR expression on LysM$^+$ macrophages and CD11c$^+$ dendritic cells was most important for the early containment of MHV within secondary lymphoid organs and to prevent lethal liver disease. This study identifies type I IFN-mediated cross-talk between plasmacytoid dendritic cells on one side and macrophages and conventional dendritic cells on the other, as an essential cellular pathway for the control of fatal cytopathic virus infection. ***The Journal of Immunology,*** **2009, 182: 1099–1106.**

For the control of fast replicating cytopathic virus infections, the immune system must act rapidly to control viral replication and dissemination before tissue damage and inflammation endanger survival of the host. Secretion of type I IFNs is an essential component of the innate immune response against viruses. These soluble factors induce an array of intracellular effectors including protein kinase R, 2′-5′-oligoadenylate synthetases and Mx proteins, which halt viral replication (1). Furthermore, type 1 IFNs exert proapoptotic activities that control viral spread by eliminating infected cells (2), and they deliver immunomodulatory stimuli that affect cell migration (3, 4), cross-presentation (5–8), B cell responses, and Ig isotype switch (9–11), CD4$^+$ T cell activation (12, 13), or CTL expansion (14, 15). However, chronic activation of the type I IFN system can be detrimental for the host because autoimmune responses might be aggravated (16, 17).

The fact that almost all cells are able to produce type I IFNs under certain conditions and also respond to it led to the initial idea of a general antiviral state. However, several in vitro studies have provided insight into the subtle differences of cell population-specific effects of type I IFNs which depend largely on the constitutive vs inducible expression of STAT proteins and IFN regulatory factors and the state of cellular differentiation (18, 19). It therefore appears that there is a cell type-specific, context-dependent differential requirement of type I IFN responsiveness that secures optimal protection against viral infection while reducing potential immunopathological side effects of these potent cytokines.

The murine hepatitis virus (MHV)[3] A59 is a group II coronavirus that causes hepatitis and demyelinating encephalomyelitis in mice. This natural mouse pathogen is one of the most extensively studied coronaviruses (20). A strong CTL response mediates clearance of the virus between days 6 and 8 postinfection (21, 22), and neutralizing Abs appear to be required to prevent re-emergence of persistent CNS infection (23, 24). Nonetheless, before effective adaptive immune responses are elicited, type I IFN-mediated innate immune responses are essential for the survival of the host in the early phase of infection. The first wave of type I IFNs is produced almost exclusively by plasmacytoid dendritic cells (pDC), leading to containment of the virus and prevention of disease (25). Thus, MHV infection represents a well-suited model to investigate whether a particular hierarchy exists in the dependency on pDC-derived type I IFNs which secure control of cytopathic viral infection and protect the host from severe disease. In this study, we have used type I IFNR-deficient (*ifnar*$^{-/-}$) bone marrow-chimeric mice and conditionally gene-targeted mice with cell type-specific IFNAR deletion to elucidate whether type I IFN signaling is required on all nucleated cells. We found that during MHV infection, the presence of the IFNAR on LysM$^+$ macrophages and CD11c$^+$ conventional dendritic cells (cDC) is of utmost importance, whereas type I IFN responsiveness of other MHV target cells such as B cells appeared not to be critical for the control of the virus. Overall, our results indicate that cells from the hemopoietic system, and in particular, macrophages and cDCs are the prime target cells for type 1 IFNs during murine coronavirus infection.

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[3] Abbreviations used in this paper: MHV, mouse hepatitis virus; pDC, plasmacytoid dendritic cell; cDC, conventional dendritic cell; IFNAR, type I IFNR; ALT, alanine 2-oxoglutarate aminotransferase.

Materials and Methods

Mice and viruses

C57BL/6 (B6) mice were obtained from Charles River Laboratories. Type I IFNR-deficient mice (*ifnar*$^{-/-}$; Ref. 26) on the B6 background were kindly provided by Dr. Martin Bachmann (Cytos, Schlieren, Switzerland) and bred in our facilities. R26-EYFP$^{+/-}$ mice (27) were kindly provided by Dr. Ari Waisman (University of Mainz, Mainz, Germany). R26-EYFP$^{+/-}$ mice and mice expressing a *loxP*-flanked *ifnar1* (*ifnar1*$^{fl/fl}$) (4) were bred with mice that express Cre recombinase specifically in B cells (CD19-Cre), T cells (CD4-Cre), T and B cells (CD19-CreCD4-Cre), macrophages, (LysM-Cre; Ref. 28), or CD11c^{+} dendritic cells (CD11c-Cre; Ref. 29). For the generation of bone marrow-chimeric mice, recipients were lethally irradiated with 900 rad from a linear accelerator (Clinic of Radio-Oncology, Kantonal Hospital St. Gallen, St. Gallen, Switzerland) and injected i.v. 1 day later with 2×10^7 of the indicated donor bone marrow cells. Chimeric mice were maintained on antibiotic water containing sulfadoxin and trimethoprim (Borgal; Veterinaria) for the following 3 wk. Mice were used for experiments 8–10 wk after bone marrow reconstitution. The degree of chimerism induced using this protocol has been routinely evaluated by reconstituting B6 mice expressing the congenic marker Thy1.2 with bone marrow cells derived from B6.Thy1.1 mice. Chimerism in these control animals was always >97%. MHV A59 was generated from a molecularly cloned cDNA (30) based on the Albany strain of MHV A59 and propagated on L929 cells. GFP-recombinant MHV was generated as previously described (31). Experiments were performed in accordance with Swiss Kantonal and Federal legislations.

Virus infections, determination of virus titers, liver enzyme values, liver histology, and IFN-α

Mice were injected i.p. with 50 PFU of MHV A59, representing a low dose infection with maximal liver disease around day 5 comparable with the kinetics of systemic infection as described previously (25). To achieve maximal target cell infection in B6 mice and minimal infection-associated death in *ifnar*$^{-/-}$ mice, a dose of 5×10^3 PFU GFP-recombinant MHV (31) was used. Intranasal infection was done with 5×10^4 PFU of MHV A59 because at this dose, 100% of the mice were reproducibly infected, and the virus did not spread systemically in B6 mice. Mice were sacrificed at the indicated time points, and organs were stored at −70°C until further analysis or disrupted for FACS analysis. Blood was incubated at room temperature to coagulate and then centrifuged; and serum was used for alanine 2-oxoglutarate aminotransferase (ALT) measurements using a Hitachi 747 autoanalyzer. Virus titers in organs were determined from frozen organs after weighing and homogenization. Viral titers were determined by standard plaque assay using L929 cells. Livers were fixed in 4% formalin and embedded in paraffin. Sections were stained with H&E. Mouse IFN-α concentration in serum or spleen homogenates was measured by ELISA (PBL Biomedical Laboratories) according to the manufacturers' instructions.

Splenocyte isolation, flow cytometry, and immunofluorescence

Splenocytes were obtained from spleens of B6, *ifnar*$^{-/-}$ or conditional EYFP mice following digestion with collagenase type II (Invitrogen) for 20 min at 37°C and resuspended in 5% RPMI 1640. For isolation of the low-density-enriched population, cells were resuspended in PBS supplemented with 2% FCS, 2 mM EDTA and overlaid on 20% Optiprep density gradient medium (Sigma-Aldrich). After centrifugation at $700 \times g$ for 15 min, low-density cells were recovered from the interface and resuspended in 5% RPMI 1640. Cells were stained with different lineage markers and analyzed for GFP expression with a FACSCalibur flow cytometer using the CellQuest software (BD Biosciences). Abs used in this study were purchased from BD Pharmingen (CD11c-PE, Ly6-G-PE (clone A8I), NK1.1-PE), Biolegend (CD4-PE, B220-allophycocyanin, CD3-allophycocyanin, CD11b-allophycocyanin), eBiosciences (F4/80-PE, CD8-PE), Miltenyi Biotec (mPDCA-1-allophycocyanin), and Immunotools (CD19-PE). For immunofluorescence analysis, spleens were immersed in HBSS and snap frozen. Five-micrometer tissue sections were fixed with acetone. Cryosections were blocked with the Fc-blocking Ab 2.4G2 and stained with the following Abs: B220 Alexa 488 (Biolegend); and F4/80 PE, CD11c-PE, and anti-MHV-N Alexa 647 (N556) kindly provided by Dr. Stuart Sidell (Department of Cellular and Molecular Medicine, University of Bristol, Bristol, U.K.). Images were acquired using a Leica DMRA microscope and processed using Adobe Photoshop (Adobe Systems).

Cell culture of primary cells and in vitro infections

Bone marrow-derived cDCs or pDCs were generated as described (25) with either GM-CSF-containing supernatant from the cell line X63-GM-CSF (kindly provided by Dr. Antonius Rolink, University of Basel, Basel, Switzerland) or Flt3-L (R&D Systems), at 20 ng/ml, respectively. cDCs were further purified using Optiprep density gradient centrifugation. pDCs were purified using the mouse pDC isolation kit (Miltenyi Biotec) adapted for the isolation of bone marrow-derived pDCs by adding CD11b-biotin (Biolegend) to the negative selection mixture. Thioglycolate-elicited macrophages were collected from the peritoneal cavity of mice and cultured overnight at 37°C. Nonadherent cells were removed by washing with ice-cold PBS. Type I IFN containing pDC supernatant was produced by infecting pDCs with MHV at a MOI of 1 for 24 h. The supernatant was filtered with Amicon Ultra 100K centrifugal filter units (Millipore) to eliminate viral particles. Sterility was confirmed by incubating supernatant on L929 cells. IFN-α concentration was determined by ELISA, and the supernatant was diluted to the indicated IFN-α concentrations. Cell survival was determined with the Cell Proliferation MTS Assay (Celltiter 96 Aqueous one-solution cell proliferation assay) from Promega. MTS solution was added to the cells 24 h postinfection. The plate was incubated for 2 h at 37°C, and the optical density was measured at 492 nm. Macrophages and cDCs were infected with MHV A59 at the indicated MOI, incubated for 1 h at 37°C, and washed. pDCs were added to the cultures after washing. A transwell plate system (BD Falcon; pore size, 0.4 μm) was used to prevent cell-cell contact between pDCs and macrophages/cDCs.

Statistical analysis

Statistical analyses were performed with Graphpad Prism 5.0 using either a nonpaired, two-tailed Student *t* test or one-way ANOVA with Bonferroni posttest comparing the samples with their corresponding control group. Survival curves were generated using the Kaplan-Meier method and the significance of differences was calculated by the log rank test. Statistical significance was defined as a value of $p < 0.05$.

Results

Early control of MHV depends on type I IFN responsiveness of hemopoietic cells

To better define the cellular targets for the activity of type I IFNs, bone marrow chimeras were generated using *ifnar*$^{-/-}$ or B6 mice. The chimeric mice that expressed the IFNAR on either hemopoietic or nonhemopoietic cells were infected i.p. with 50 PFU of MHV A59. Because *ifnar*$^{-/-}$ mice succumb to MHV infection rapidly (25), mice were sacrificed after 48 h, and IFN-α production, severity of liver disease, and viral titers in spleens, livers, and lungs were determined. As shown in Fig. 1*A*, neither the lack of the IFNAR on hemopoietic nor that on nonhemopoietic cells precluded production of IFN-α. Furthermore, induction of IFN-β was not influenced by the absence of IFNAR on different cell subsets (data not shown). The lack of the IFNAR on radio-resistant parenchymal cells (B6→*ifnar*$^{-/-}$) did not lead to significantly elevated liver enzyme values, whereas the absence of the IFNAR on bone marrow-derived cells (*ifnar*$^{-/-}$→B6) resulted in severe liver disease (Fig. 1*C*). Moreover, viral titers in livers, spleens, and lungs (Fig. 1*B*) from these mice were significantly higher than in mice that expressed the IFNAR only on hemopoietic cells. Most importantly, the expression of IFNAR on the hemopoietic cells (B6→*ifnar*$^{-/-}$) secured significantly longer survival of the mice (Fig. 1*D*). These results indicate a clear hierarchy in the importance of the IFNAR expressed on hemopoietic vs nonhemopoietic cells; the presence of the IFNAR on hemopoietic cells appears to be important to contain the virus within secondary lymphoid organs and thereby contributes critically to the prevention of disease.

Target cells of MHV within the bone marrow-derived cell compartment

It is likely that those cells that are most easily infected by a cytopathic virus and therefore rapidly lost during the infection are most dependent on the protection provided by the type I IFN system. Working along this assumption, we first determined which cell

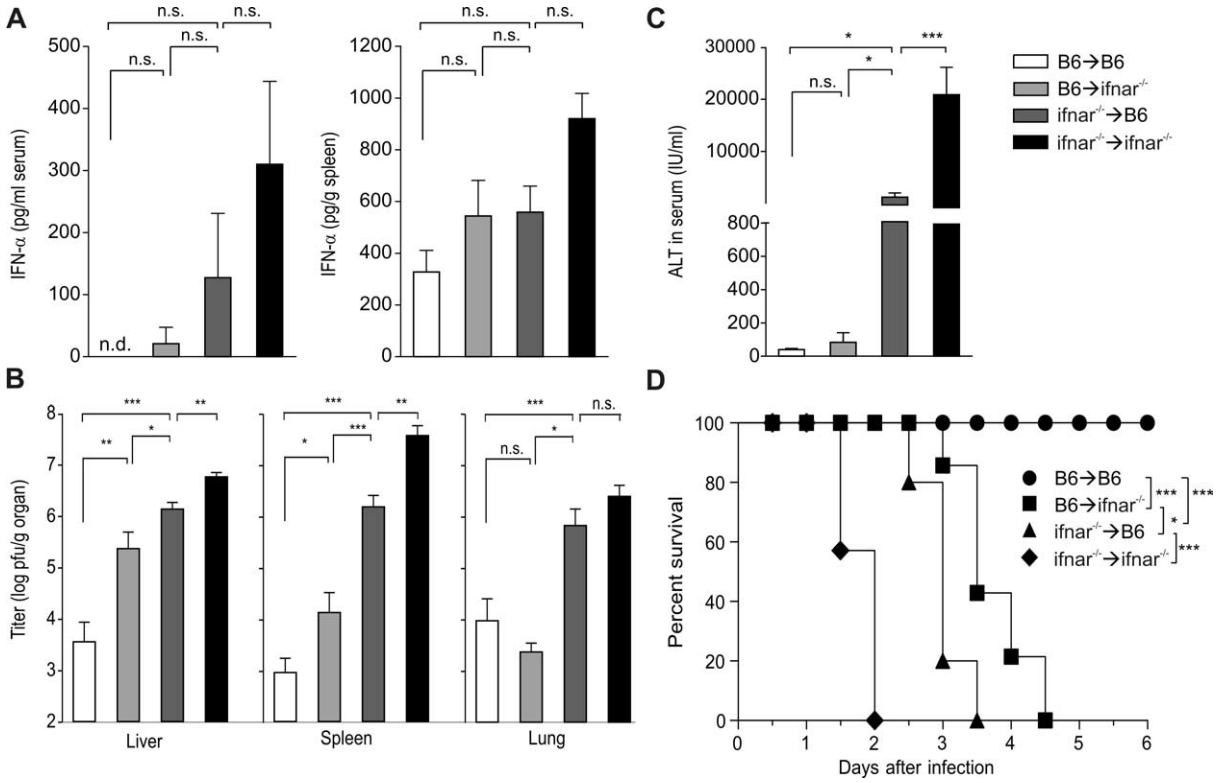

FIGURE 1. Type I IFN responsiveness of bone marrow-derived cells is essential for early control of MHV infection. Bone marrow-chimeric mice (B6→*ifnar*$^{-/-}$, *ifnar*$^{-/-}$→B6, B6→B6, *ifnar*$^{-/-}$→*ifnar*$^{-/-}$) were infected i.p. with 50 PFU of MHV A59. After 48 h, IFN-α concentration in serum and spleens (*A*); viral titers in livers, spleens, and lungs (*B*); ALT values in serum (*C*) were determined. Results represent means ± SD of five to six mice per group. *D*, Survival of bone marrow-chimeric mice. Health status was monitored twice daily, and moribund animals were euthanized (n = 5–6 mice per group). Statistical analysis was performed using one-way ANOVA with Bonferroni posttest. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$, n.s., $p > 0.05$. n.d., Not detected. Survival curves were generated using the Kaplan-Meier method, and the significance of differences was calculated by the log-rank test. Statistical significance was defined as ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$, n.s., $p > 0.05$.

populations within the hemopoietic compartment support MHV infection. In a first set of experiments, splenocytes from B6 or *ifnar*$^{-/-}$ mice were infected in vitro with GFP-recombinant MHV at a MOI of 1. After 12 h, MHV replication in macrophages (F4/80$^+$CD11b$^+$), neutrophils (Ly6G$^+$CD11b$^+$), cDCs (CD11c$^+$B220$^-$), B cells (CD19$^+$), CD4$^+$ T cells (CD3$^+$CD4$^+$), and CD8$^+$ T cells (CD3$^+$CD8$^+$) was determined by flow cytometry (Fig. 2*A*). This analysis revealed that primary macrophages, cDCs, neutrophils, and B cells could be infected with MHV and that the lack of IFNAR on these cells slightly increased their susceptibility. To confirm whether this target cell tropism of MHV for particular leukocyte subsets can be reproduced in vivo, B6 and *ifnar*$^{-/-}$ mice were infected with 5×10^3 PFU of GFP-recombinant MHV i.p., and the different spleen cell populations were probed for GFP expression 36 h postinfection using flow cytometric analysis as described previously. We could not detect GFP-positive cells in the different splenocyte fractions derived from infected B6 mice (Fig. 2*B, top row*), suggesting that the intact type I IFN system in these mice had efficiently blocked viral replication below the level of detection. Indeed, macrophages, cDCs, B cells, and neutrophils from infected *ifnar*$^{-/-}$ mice showed significant GFP expression (Fig. 2*B*, *bottom row*). Other leukocyte populations such as CD4$^+$ and CD8$^+$ T lymphocytes (Fig. 2*B*) and NK cells (not shown) did not exhibit significant GFP expression. Furthermore, B6 and *ifnar*$^{-/-}$ mice were infected with 5×10^3 PFU of MHV, and fluorescence microscopic analysis was performed using anti-MHV nucleoprotein Ab to identify infected cells in situ. Whereas MHV-infected F4/80$^+$ cells in the red pulp (Fig. 2*C*) and CD11c$^+$ in the white pulp (Fig. 2*D*) could be readily detected in spleens of *ifnar*$^{-/-}$ mice, colocalization of the MHV nucleoprotein with the B cell marker B220 (Fig. 2, *C* and *D*) and with the neutrophil marker Ly6G (not shown) was rare. As expected, only very few MHV-infected cells were found in B6 mice (not shown), thus confirming the high susceptibility of cDCs and macrophages to MHV infection in the absence of a functional type I IFN system.

Requirement of IFNAR expression on different leukocyte populations

To assess the differential requirement of type I IFN responsiveness of the MHV target populations, we used a set of conditionally gene-targeted mice. Crossing of mice with a *loxP*-flanked *ifnar1* (*ifnar1*$^{fl/fl}$) with mice that express the Cre recombinase in a cell type-specific manner resulted in deletion of the IFNAR in T cells (CD4-Cre$^{+/-}$*ifnar*$^{fl/fl}$) (4), in B cells (CD19-Cre$^{+/-}$*ifnar1*$^{fl/fl}$) (4), in T and B cells (CD4-Cre$^{+/-}$CD19-Cre$^{+/-}$*ifnar1*$^{fl/fl}$), in macrophages, neutrophils, and some dendritic cells (LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$; Ref. 28), and specifically in CD11c$^+$ cDCs (CD11c-Cre$^{+/-}$*ifnar1*$^{fl/fl}$; Ref. 29). These mice were infected with MHV, and survival was monitored for 2 wk. As shown in Fig. 3*A*, the expression of the IFNAR on the surface of LysM$^+$ or CD11c$^+$ cells was essential for survival, since LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ and CD11c-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ mice succumbed to the infection. LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ developed a more severe phenotype with lethal disease after 4 days of infection. Likewise, LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ mice showed the most severe liver pathology with significantly elevated ALT values as early as day 2 postinfection (Fig. 3*B*) and a massive damage of liver tissue (Fig. 3*C*). Because neutrophils can be infected with MHV in vivo (Fig. 2*B*), we determined next whether the presence of neutrophils in LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$

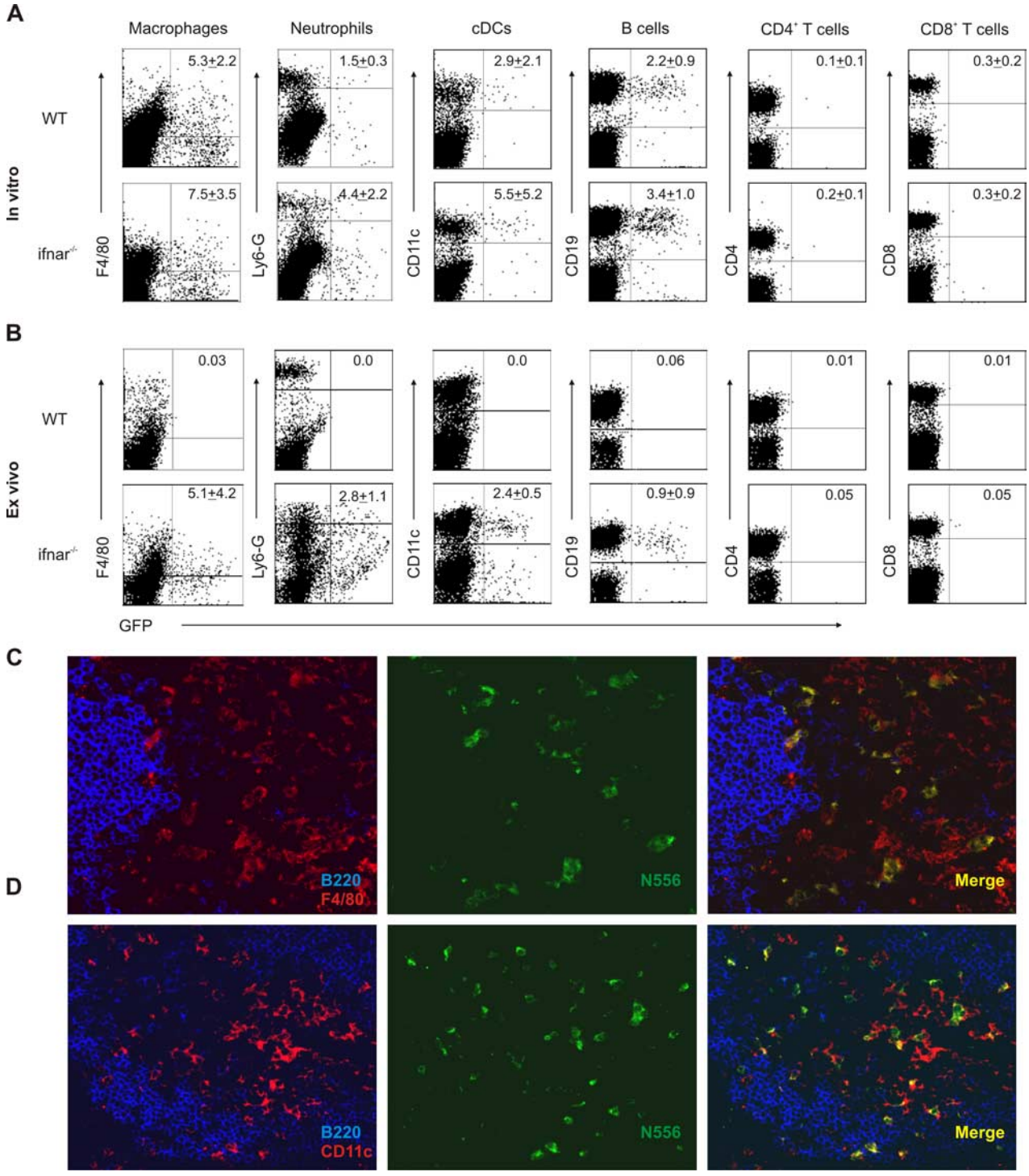

FIGURE 2. MHV target cells in vitro and in vivo. *A*, 10^6 splenocytes or low-density cell-enriched fractions (for cDC analysis) from *ifnar*$^{-/-}$ or B6 mice were infected with GFP-recombinant MHV (MOI 1). Cells were harvested 12 h later and stained for the indicated surface molecules (macrophages, F4/80$^+$, CD11b$^+$; neutrophils, Ly6G$^+$CD11b$^+$; cDCs, B220$^-$CD11c$^+$; B cells, CD19$^+$; CD4 T cells, CD4$^+$CD3$^+$; CD8 T cells, CD8$^+$CD3$^+$. *B*, *ifnar*$^{-/-}$ or B6 mice were infected i.p. with 5×10^3 PFU of GFP-recombinant MHV. Spleens were collected after 36 h and digested with collagenase, and splenocytes or low-density cells (for cDCs analysis) were stained for the indicated cell population as in *A*. Dot plots are representative of five individual mice. Numbers in the upper right quadrant indicate mean percentages ± SD of GFP$^+$ cells for each population. *C* and *D*, Representative sections from the spleens of *ifnar*$^{-/-}$ mice infected with 5×10^3 PFU of MHV A59 24 h postinfection. Staining: B220, blue; MHV-N, green; F4/80, red in *C*; CD11c, red in *D*. WT, Wild type. Original magnification, ×400.

could affect viral distribution and virus-mediated disease. To this end, neutrophils were depleted in LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ and B6 mice using the NIMP-R14 Ab (32). NIMP-R14-mediated depletion of neutrophils in B6 mice had no significant effect on MHV replication in the major target organs (data not shown). Likewise, MHV replication and infection-associated hepatitis was not affected by the absence of neutrophils in LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ mice (Fig. 3*D*), indicating that in these mice it is the absence of the IFNAR on macrophages, not on neutrophils, that determines the high susceptibility to MHV infection. CD11c-Cre$^{+/-}$*ifnar1*$^{fl/fl}$

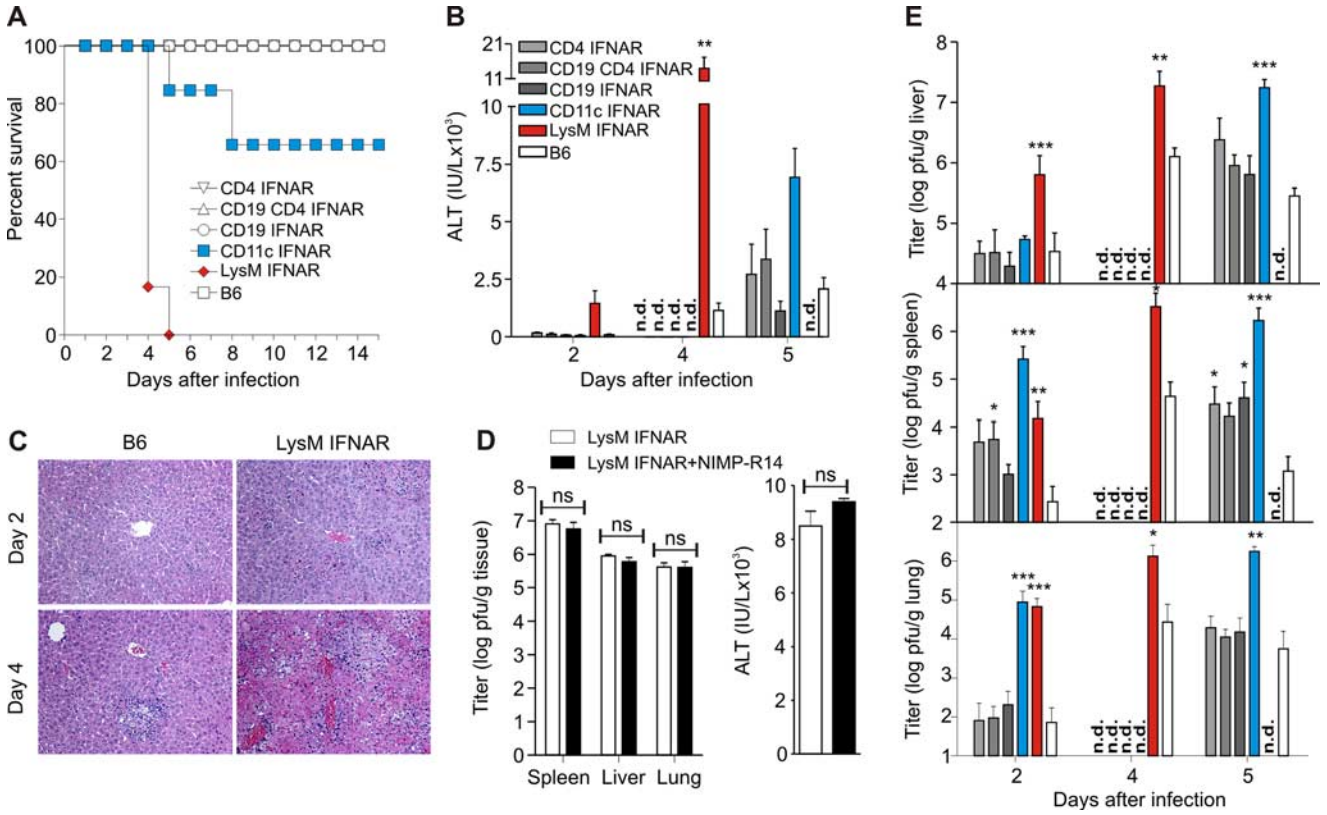

FIGURE 3. Requirement for IFNAR expression on different leukocyte subsets. CD4-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, CD4-Cre$^{+/-}$CD19-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, CD19-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, CD11c-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, and B6 mice were infected i.p. with 50 PFU of MHV A59. *A*, Survival of conditionally IFNAR$^-$ mice. Health status was monitored twice daily, and moribund animals were euthanized (n = 6–7 mice per group). *B*, ALT values in serum were determined at the indicated time points postinfection. *C*, Liver pathology in LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ and B6 mice on days 2 and 4 postinfection. H&E staining. *D*, MHV replication in neutrophil-depleted LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ mice. LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ mice were injected with 250 μg of NIMP-R14 Ab and 24 h later were infected with 50 PFU of MHV A59. At day 2 postinfection, a second NIMP-R14 injection was administered. Neutrophil depletion (>95%) was confirmed by flow cytometry. Viral titers in livers, spleens, and lungs (*left*) and ALT values in serum (*right*) were determined at day 4 postinfection. Results represent means ± SD of three mice per group. Statistical analysis was performed using Student's t test. *E*, Viral titers in livers, spleens, and lungs were determined at the indicated time points postinfection. Results represent means ± SD of five mice per group. Statistical analysis was performed using one-way ANOVA with Bonferroni posttest. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ comparing the values from the corresponding conditional IFNAR$^-$ mice with B6 values at the same day. n.d., not determined.

mice exhibited a slightly delayed onset of liver disease with peak values at about day 5 postinfection (Fig. 3*B*). Mice lacking the IFNAR on T and/or B cells showed no exacerbation of MHV-induced liver disease (Fig. 3*B*). The clear hierarchy of cell type-dependent, type I IFN-mediated protection from disease correlated well with viral replication observed in livers, spleens, and lungs (Fig. 3*E*). Clearly, mice lacking the IFNAR on macrophages in LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ mice were most susceptible to MHV infection resulting in uncontrolled spread through all organs. CD11c-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ mice were, as well, highly susceptible with particularly strong replication in spleens supporting the notion that splenic cDCs represent a major target cell population of MHV within this organ. Although B cells could be infected with MHV in vitro (Fig. 2*A*) and were found to be infectable in *ifnar*$^{-/-}$ mice in vivo (Fig. 2*B*), the specific IFNAR deficiency on B cells only moderately influenced viral replication (Fig. 3*E*), which is probably related to the poor capacity of MHV-infected B cells to produce viral particles in comparison with cDCs or macrophages (data not shown). Likewise, CD4-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ and CD4-Cre$^{+/-}$CD19-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ mice showed only mildly increased susceptibility to MHV infection. All mice that survived until day 15 postinfection had cleared the virus, including the remaining CD11c-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ mice (data not shown), suggesting that the adaptive immune system had successfully contained the viral infection.

To evaluate the importance of IFN-α/β production for different target cell populations following a peripheral route of infection, CD4-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, CD19-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, CD11c-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, and B6 mice were infected intranasally with 5×10^4 PFU of MHV, and the severity of the disease, viral distribution, and viral titers were determined on day 6 postinfection. As shown in Fig. 4*A*, mice lacking the IFNAR on LysM$^+$ and CD11c$^+$ cells developed severe liver disease, whereas the absence of the IFNAR on T and B cells did not precipitate an elevation in liver enzyme values. Comparable with the results from the systemic (i.p.) infection, type I IFN responsiveness by macrophages was most important for the control of the virus (Fig. 4*B*). Also, the lack of the IFNAR on B or T cells was of importance for the systemic spread of the virus which was still detectable in the liver at substantial titers (Fig. 4*B*). The finding that the presence or absence of this receptor on different cell populations did not influence the ability of the virus to enter the CNS (Fig. 4*B*) illustrates the context-dependent and organ-specific importance of IFNAR expression.

Conditional targeting using the Cre/*loxP* system permits functional assessment of particular molecules in certain cell types. However, absolute cell type specificity can usually not be achieved

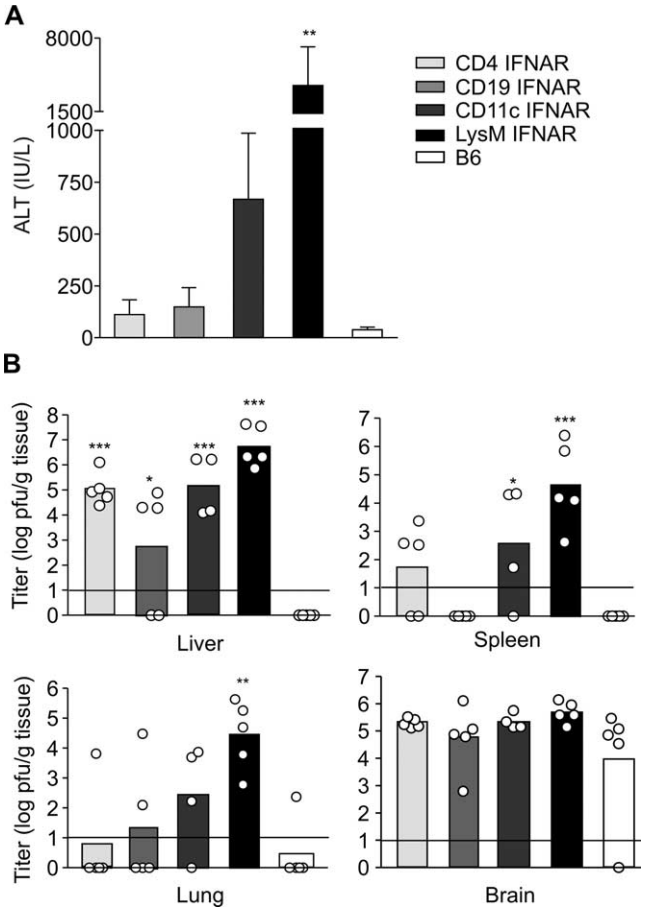

FIGURE 4. Type I IFN-dependent control of MHV following intranasal infection. CD4-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, CD19-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, CD11c-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ and B6 mice were infected with 5×10^4 PFU MHV A59. Six days postinfection, ALT values in serum (*A*) and viral titers in livers, spleens, lungs, and the CNS (*B*) were determined. Bars, means; ○, values from individual mice. Statistical analysis was performed using one-way ANOVA with Bonferroni posttest (***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ comparing the values from the corresponding conditional IFNAR-deficient mice with B6 values at the same day).

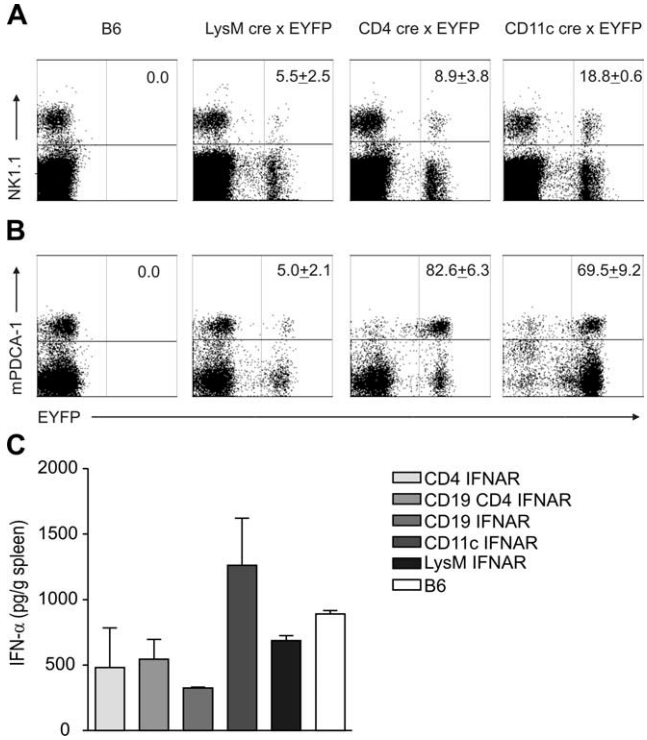

FIGURE 5. Cre recombinase-driven gene recombination in NK cells, pDCs, and type I IFN responsiveness. Cre recombinase activity in CD3$^-$NK1.1$^+$ NK cells (*A*) and CD11c$^+$mPDCA-1$^+$ pDCs (*B*) was analyzed by the expression of the *EYFP* reporter gene in B6, LysM-Cre$^{+/-}$R26-EYFP$^{+/-}$, CD4-Cre$^{+/-}$R26-EYFP$^{+/-}$, and CD11c-Cre$^{+/-}$R26-EYFP$^{+/-}$ mice. Dot plots show analysis of one representative of six individual mice. Numbers in the upper right quadrant indicate mean percentages ± SD of EYFP$^+$ cells for each population. *C*, IFN-α production in the different conditionally IFNAR$^-$ mice. CD4-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, CD4-Cre$^{+/-}$CD19-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, CD19-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, CD11c-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, LysM-Cre$^{+/-}$*ifnar1*$^{fl/fl}$, and B6 mice were infected i.p. with 50 PFU of MHV A59. Forty-eight hours after infection, IFN-α concentration in spleen homogenates was determined by ELISA (mean ± SD, $n = 2$–4 mice).

by this approach. We thus performed a side-by-side comparison of different Cre driver mice which allowed us to extract the relevant information from a more complex data set. NK cells and pDCs are critical during the early phase of a viral infection. To analyze the Cre recombinase activity in these cell populations, we crossed CD4-Cre, CD11c-Cre, and LysM-Cre mice with the R26-EYFP strain which permits detection of the *EYFP* reporter gene in those cells with active Cre recombinase (27). Moreover, to address the question of whether deletion of the IFNAR on pDCs in the conditionally gene-targeted mice might have influenced the overall type I IFN responsiveness to MHV infection, we determined IFN-α production in the conditionally *ifnar*$^{-/-}$ mice. Cre recombinase was active in only a few NK1.1$^+$ cells from LysMCre$^{+/-}$R26-EYFP$^{+/-}$ or CD11c-Cre$^{+/-}$R26-EYFP$^{+/-}$ mice (Fig. 5*A*). Furthermore, given that 10% of NK1.1$^+$ cells in CD4-Cre$^{+/-}$R26-EYFP$^{+/-}$ mice were EYFP$^+$ and CD4-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ mice did not show a significant impairment in their susceptibility to MHV (Fig. 3), such a small proportion of IFNAR$^-$ NK cells did most likely not influence susceptibility to MHV infection. Cre recombinase activity could be readily detected in mPDCA-1$^+$ pDCs from CD11c-Cre$^{+/-}$R26-EYFP$^{+/-}$ (Fig. 5*B*) as described previously (29). The finding that CD11c-Cre$^{+/-}$*ifnar1*$^{fl/fl}$ mice responded with vigorous IFN-α production to MHV infection (Fig. 5*C*) suggested that the lack of the IFNAR on pDCs had no significant impact on the early type I IFN response. This notion is supported by the findings that >82% of the pDCs were EYFP$^+$ in CD4-Cre$^{+/-}$R26-EYFP$^{+/-}$ mice (Fig. 5*B*), which controlled MHV infection efficiently (Fig. 3), and that IFN-α production was not impaired in these mice (Fig. 5*C*). Taken together, these data indicate that the absence of the IFNAR on pDCs did not affect the resistance to MHV infection in conditionally *ifnar*$^{-/-}$ mice.

Protection of macrophages and dendritic cells by pDC-derived IFN-α

Taken together, the data presented above indicated that macrophages and cDCs are most dependent on the protection provided by the type I IFN system and that this stimulation is necessary to secure control of systemic MHV infection. To provide insight into the mechanisms underlying the type I IFN-induced antiviral state in these two important target cell populations, a series of in vitro experiments were performed. Virus-free cell culture supernatant from MHV-infected pDCs containing defined amounts of IFN-α was used to estimate the protective capacity of pDC-derived IFN-α for both cDCs or macrophages. pDC-derived type I IFN significantly reduced cell death of cDCs (Fig. 6*A*) and macrophages (Fig. 6*B*) when the cells were exposed to high doses of virus (MOI 1).

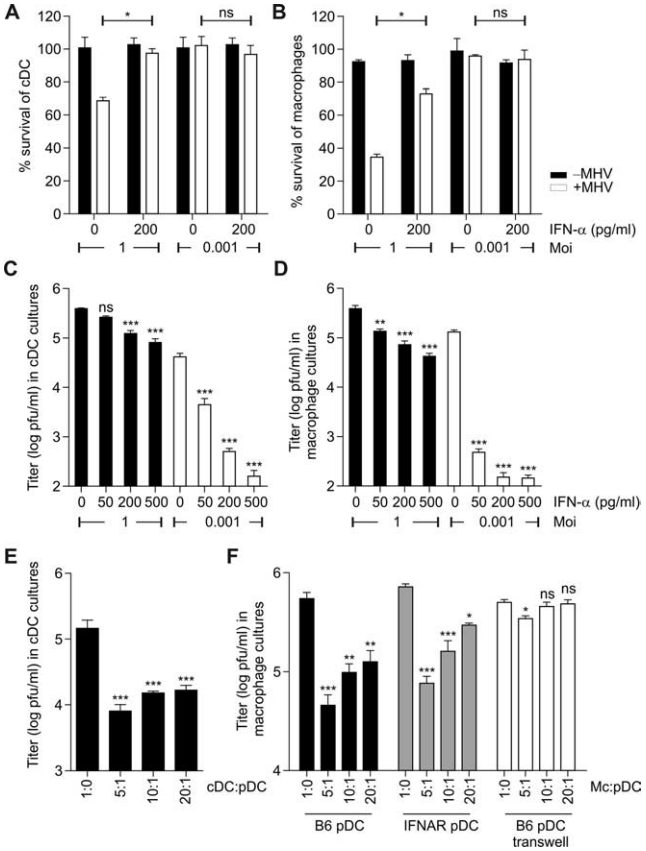

FIGURE 6. Effect of pDC-derived type I IFN on cDC and macrophage (Mc) survival and control of MHV replication. Five $\times$ 10^4 cDCs (*A*) or macrophages (*B*) were preincubated for 5 h with pDC supernatant containing 0 or 200 pg/ml IFN-α and infected with MHV A59 at a MOI of 1 or 0.001. Survival was analyzed after 24 h using the MTS assay. Statistical analysis was performed using Student's *t* test (***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$, n.s., $p > 0.05$). Five $\times$ 10^5 cDCs (*C*) or macrophages (*D*) were preincubated with pDC supernatant containing the indicated concentrations of IFN-α and infected with MHV A59 at a MOI of 1 or 0.001. Five $\times$ 10^5 cDCs (*E*) or macrophages (*F*) were infected with MHV A59 at a MOI of 0.001. B6 or *ifnar*$^{-/-}$ pDCs were added directly or in a transwell to the infected cells at the indicated ratio. Viral titers in the supernatants were analyzed after 24 h. Bars, Means $\pm$ SD of quadruplicate samples from one of three independent experiments. Statistical analysis was performed using one-way ANOVA with Bonferroni posttest. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$, n.s., $p > 0.05$ comparing the values from the corresponding column with the unpretreated control (*C* and *D*) or the cells without addition of pDCs (*E* and *F*).

Likewise, pDC-derived type I IFN significantly reduced MHV production in both target cell populations (Fig. 6, *C* and *D*). Incubation of *ifnar*$^{-/-}$ macrophages and cDCs with pDC-derived supernatant did neither reduce MHV-induced cell death nor MHV replication (data not shown) indicating that the observed protective effect depended solely on signals transmitted via the IFNAR. Furthermore, cocultures of uninfected pDCs with infected cDCs (Fig. 6*E*) and macrophages (Fig. 6*F*) revealed that only few pDCs were sufficient to protect the adjacent target cells. pDC-mediated target cell protection was not dependent on the presence of the IFNAR on pDCs (Fig. 6*F, middle*). However, viral replication in IFNAR-competent cells was not halted when pDCs and macrophages were separated in transwell chambers, indicating that infected target cells and type I IFN-producing pDCs had to be in close vicinity to permit access of pDCs to viral particles or viral compounds that could trigger the protective IFN response in pDCs. (Fig. 6*F, right*). Overall, these data revealed that the IFNAR on macrophages and cDCs together with its stimulation by pDC-derived type I IFN is essential to prevent excessive viral replication in the target cells and to secure survival of these important APCs.

Discussion

A major function of both macrophages and cDCs during viral infections is their instructive role for the developing adaptive immune response. Macrophages in the marginal sinuses of lymph nodes, for example, are able to collect Ag from the incoming lymph stream and present Ag to follicular B cells (33, 34). Marginal zone macrophages in spleen can bind viruses decorated by complement and natural Abs and reduce thereby dissemination of viruses to peripheral organs (35). This trapping of viral particles on macrophages is important to enhance the induction of protective T cell responses (36). It has been shown that the enhanced binding of viral particles to macrophages also fosters their infection (37). Likewise, cDCs can be infected with essentially all viruses irrespective of their tissue tropism. The high susceptibility of cDCs to viral infection appears to be important for the efficient direct priming of CTL (38). The results of this study emphasize the importance of type I IFN-mediated protection of both macrophages and cDCs. The lack of the IFNAR on macrophages in LysM-Cre$^{+/-}$ *ifnar1*$^{fl/fl}$ mice led to completely uncontrolled viral replication and death in only 4 days. Furthermore, the absence of the IFNAR on cDCs resulted in death of ~40% of the animals between days 6 and 8. At this time, the CTL response is supposed to clear the virus infection. Thus, type I IFNs provide protection of two highly vulnerable cell populations and therefore facilitate 1) removal of the virus from the circulation by macrophages and 2) preservation of cDC integrity for the priming of adaptive immune responses.

MHV is a rapidly replicating virus exhibiting a high cytopathicity that leads to severe inflammation in several organs (39). Systemic virus infection with dissemination via the bloodstream into visceral organs has been mimicked in this study by i.p. application. Our results show that type I IFN responsiveness in macrophages and cDCs is necessary to prevent severe liver disease and to secure survival of the host. However, MHV may escape immunosurveillance and establish chronic infection in the CNS, leading to progressive demyelinating disease (20). MHV can enter the CNS via the olfactory nerve system (40) and spreads transneuronally, leading to infection of distinct parts of the brain and the spinal cord. CTLs control viral replication within the CNS but cannot completely eliminate the virus (21), whereas neutralizing Abs are essential to prevent viral recrudescence (24). Thus, MHV is well adapted to use the CNS as an immunoprivileged site to escape complete clearance from the system. Part of that escape strategy may be the inability of the type I IFN system to prevent spread of the virus to the CNS, as shown in this study. Intranasal inoculation which permits direct access of the virus to olfactory nerve endings, facilitated efficient neuroinvasion of the virus irrespective of the presence of absence of the IFNAR. Direct intracranial application of a gliatropic strain of MHV results in a severely accelerated lethal CNS disease even in the presence of fully function antiviral CD8$^+$ T cells (41), indicating that the type I IFN system also contributes to the control of viral dissemination within the CNS. It will be important in future studies to determine which cell type (neuron, glia, or hemopoietic) is critical for the type I IFN-mediated containment of MHV in this immunoprivileged site.

Viruses have developed a remarkable array of countermeasures to interfere with the type I IFN system. Coronaviruses, despite generation of significant amounts of type I IFN inducing dsRNA, are able to suppress early IFN-β induction (42, 43). Furthermore, immunomodulatory nonstructural proteins (Nsp) such as Nsp1 are

able to inhibit IFN-α responsiveness in a cell type-specific manner (31). Thus, cDCs and macrophages, which fail to raise significant IFNα responses following coronavirus infection (25), are particularly dependent on the external supply of protective type I IFNs. It appears that during coronavirus infections, it is the pDC-derived type I IFN (25) that provides protection for those infected cells that are otherwise incapacitated by particular viral proteins. Taken together, our study provides insight into the context-dependent regulation of the type I IFN system and highlights the importance of type I IFN-mediated cross-talk between pDCs and cDCs/macrophages which most likely represents an essential cellular pathway for the protection against cytopathic virus infections.

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Disclosures

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