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Inflammatory monocytes and NK cells play a crucial role in DNAM-1–dependent control of cytomegalovirus infection

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The poliovirus receptor (PVR) is a ubiquitously expressed glycoprotein involved in cellular adhesion and immune response. It engages the activating receptor DNAX accessory molecule (DNAM)–1, the inhibitory receptor TIGIT, and the CD96 receptor with both activating and inhibitory functions. Human cytomegalovirus (HCMV) down-regulates PVR expression, but the significance of this viral function in vivo remains unknown. Here, we demonstrate that mouse CMV (MCMV) also down-regulates the surface PVR. The m20.1 protein of MCMV retains PVR in the endoplasmic reticulum and promotes its degradation. A MCMV mutant lacking the PVR inhibitor was attenuated in normal mice but not in mice lacking DNAM-1. This attenuation was partially reversed by NK cell depletion, whereas the simultaneous depletion of mononuclear phagocytes abolished the virus control. This effect was associated with the increased expression of DNAM-1, whereas TIGIT and CD96 were absent on these cells. An increased level of proinflammatory cytokines in sera of mice infected with the virus lacking the m20.1 and an increased production of iNOS by inflammatory monocytes was observed. Blocking of CCL2 or the inhibition of iNOS significantly increased titer of the virus lacking m20.1. In this study, we have demonstrated that inflammatory monocytes, together with NK cells, are essential in the early control of CMV through the DNAM-1–PVR pathway.

INTRODUCTION

Cytomegaloviruses (CMVs) are species-specific herpesviruses causing severe disease in immunocompromised and immunologically immature hosts. Mouse CMV (MCMV) is biologically similar to human CMV (HCMV), and therefore serves as a widely used model for studying CMV pathogenesis (Reddehase, 2002). Cells of the innate immune system play a crucial role in cytomegaloviral control before the initiation of specific immunity (Vidal et al., 2013). NK cells represent an essential component of innate immunity as a result of their ability to identify infected cells via a set of signals provided by activating and inhibitory receptors (Shifrin et al., 2014). The mononuclear phagocyte system is composed of monocytes, macrophages, and DCs. Monocytes are highly adaptable cells that can differentiate into monocyte-derived macrophages and monocyte-derived DCs (Chow et al., 2011). Macrophages are professional phagocytic cells whose main function is to inactivate

and destroy invading pathogens (Martinez and Gordon, 2014). A direct macrophage infection in lymph node results in limiting CMV spread (Farrell et al., 2015). Following their genetic programs, instructed in part by their tissue microenvironment and by the signals gathered through their receptors, mononuclear phagocytes can adopt a variety of specific functional programs, encompassing, but not limited to, the well-known M1 versus M2 phenotypes (Italiani and Boraschi, 2014; Murray et al., 2014). The M1, with its proinflammatory features, is protective against viruses and other intracellular parasites. This phenotype is associated with the production of proinflammatory cytokines such as IFN- γ or IL-12 and activation of inducible nitric oxide synthase (iNOS)–NO pathway. Alternatively, mononuclear phagocytes can polarize to M2 cells associated with IL-4 and arginase production. Although the polarization of mononuclear phagocytes may be essential for ultimate virus control, the mechanisms used by various viruses to regulate this cellular programming are still insufficiently characterized.

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Abbreviations used: BAC, bacterial artificial chromosome; BMDC, BM-derived DC; CMV, cytomegalovirus; DNAM, DNAX accessory molecule; HCMV, human CMV; MCMV, mouse CMV; MM, marginal metallophilic macrophage; MZ, marginal zone macrophage; PVR, poliovirus receptor; RP, red pulp macrophage.

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The poliovirus receptor (PVR or CD155), a member of the nectin protein family, serves as a ligand for the adhesion molecule DNAX accessory molecule 1 (DNAM-1; CD226; Shibuya et al., 1996; Bottino et al., 2003). DNAM-1 is an activating receptor expressed on the majority of immune cells, including monocytes, T cells, NK cells, and as a subset of B cells (Shibuya et al., 1996; Bottino et al., 2003; Chan et al., 2014; de Andrade et al., 2014; Vo et al., 2016). Upon recognition of its ligands, CD155 (PVR) and CD112 (Nectin-2), DNAM-1 promotes NK cell activation and elimination of infected cells (de Andrade et al., 2014). Recent data revealed that DNAM-1 expression marks an alternative maturation program of NK cells (Martinet et al., 2015) and plays a role in the generation of memory NK cells (Nabekura et al., 2014). However, the role of DNAM-1 in virus control by various subsets of mononuclear phagocytes has not been so far established. PVR is also a high affinity ligand for TIGIT, a receptor that inhibits NK and T cell cytotoxicity (Stanitsky et al., 2009, 2013; Yu et al., 2009; Joller et al., 2011; Levin et al., 2011). Moreover, PVR binds to the CD96 (Tactile) receptor with both activating and inhibitory functions on NK cells (Fuchs et al., 2004; Chan et al., 2014). The functional outcome of a simultaneous PVR ligation of activating and inhibitory receptors on immune cells and virus control is therefore hard to predict. This becomes even more evident if we consider that PVR is expressed on the majority of somatic cells under physiological conditions and that its expression is induced as a consequence of viral infections and tumorigenesis (Chadéneau et al., 1994; Groemeier et al., 2000; Masson et al., 2001; Hirota et al., 2005; Tomasec et al., 2005; Magri et al., 2011; Vassena et al., 2013; Nabekura et al., 2014). Cells up-regulate PVR expression in response to Ras activation and DNA damage response pathway, as well as Toll-like receptor activation (Hirota et al., 2005; Soriani et al., 2009; Kamran et al., 2013; Vassena et al., 2013).

HCMV encodes a protein that reduces PVR surface expression on infected cells (Tomasec et al., 2005), but the impact of this viral function on virus control *in vivo* could not be addressed due to the strict species specificity of HCMV. To overcome this limitation and assess the relevance of viral regulation of PVR for virus control, we have used the infection of mice with MCMV. We have observed that, similar to HCMV, MCMV also retains PVR inside of infected cells, and we have characterized the viral gene involved. The deletion of the MCMV inhibitor of PVR dramatically enhances the virus susceptibility to innate immune control in DNAM-1–dependent manner. This function is partially dependent on NK cells that express both inhibitory and activating PVR receptors, but the virus control also strongly depends on mononuclear phagocytes that display a dramatic increase of DNAM-1 expression upon infection and, at the same time, fail to express inhibitory PVR receptors.

RESULTS

MCMV retains PVR in infected cells and prevents its surface expression

Our first goal was to assess whether MCMV, like HCMV, interferes with the PVR expression. Although the transcrip-

tion of PVR during MCMV infection was increased (Fig. 1, A and B, left; Juranic Lisnic et al., 2013), and WT MCMV infection resulted in up-regulation of PVR transcript compared with mock-infected control, we found the surface level of PVR to be reduced upon MCMV infection of primary MEF (Fig. 1, B [right] and C). Similar down-regulation of surface PVR was confirmed on several cell lines infected with MCMV (Fig. 1 D). In contrast, UV inactivated virus did not down-regulate PVR, and the kinetics of PVR reduction suggested that a viral gene product expressed early after infection is responsible for PVR down-regulation (unpublished data).

Next, we analyzed the molecular mechanism involved in viral PVR down-regulation. The molecular mass of the mature PVR protein in uninfected cells is between 80 and 90 kD (Fig. 2 A). However, the infection with MCMV resulted in the accumulation of a lower molecular form of PVR of ~70 kD (Fig. 2 A). The treatment of lysates of MCMV-infected cells with EndoH revealed that the 70-kD protein form is EndoH sensitive, implying that MCMV retains PVR inside the cell (Fig. 2 B). Inhibitors of cellular degradation pathways were tested, and increased PVR amounts were seen in infected cells treated with lactacystin, an inhibitor of proteasomal degradation, whereas the lysosomal inhibitor leupeptin had minimal or no effect (Fig. 2 C). The dominance of the proteasomal degradation was further confirmed by confocal microscopy (Fig. 2 D). These data show that, similar to HCMV, MCMV retains PVR in the ER, prevents its maturation, and initiates its degradation in the proteasome.

Recently, it has been shown that DCs and macrophages up-regulate PVR expression upon MCMV infection (Nabekura et al., 2014). To check whether MCMV also retains the PVR in cells other than primary fibroblasts, different cell lines were infected with WT MCMV, and their lysates were analyzed for PVR retention. As shown in Fig. 2 E, the PVR retention was evident in all cell lines tested, including BM-derived DCs (BMDCs), the DC cell line DC2.4, and macrophage cell line J774. Testing the PVR retention in cells infected with field isolates of MCMV (Smith et al., 2008) showed the same PVR retention phenomenon (Fig. 2 F). Thus, PVR retention is conserved between various MCMV strains and functional in different cell types.

Characterization of the MCMV protein involved in PVR regulation

The next goal was to identify and characterize MCMV genes involved in PVR retention. Using a library of MCMV mutants with genomic deletions, we could show that the gene responsible for the PVR retention is located in the *m01–m22* gene region ($\Delta 8$ virus, Fig. 3 A). Because PVR maturation in cells infected with an MCMV mutant lacking the segment of first 17 genes ($\Delta 1$ virus) was comparable with the WT MCMV, we concluded that the PVR regulator must lie in the *m18–m22* gene region (Fig. 3 A). To determine the role of individual genes in this region, according to previously annotated ORFs (Rawlinson et al., 1996), we constructed MCMV mutants with deletions in the genes *m18*, *m19*, *m21*, or *m22* (Fig. 3 B, top). Because ORF *m20* significantly overlaps with ORFs *m19* and *m21*, it

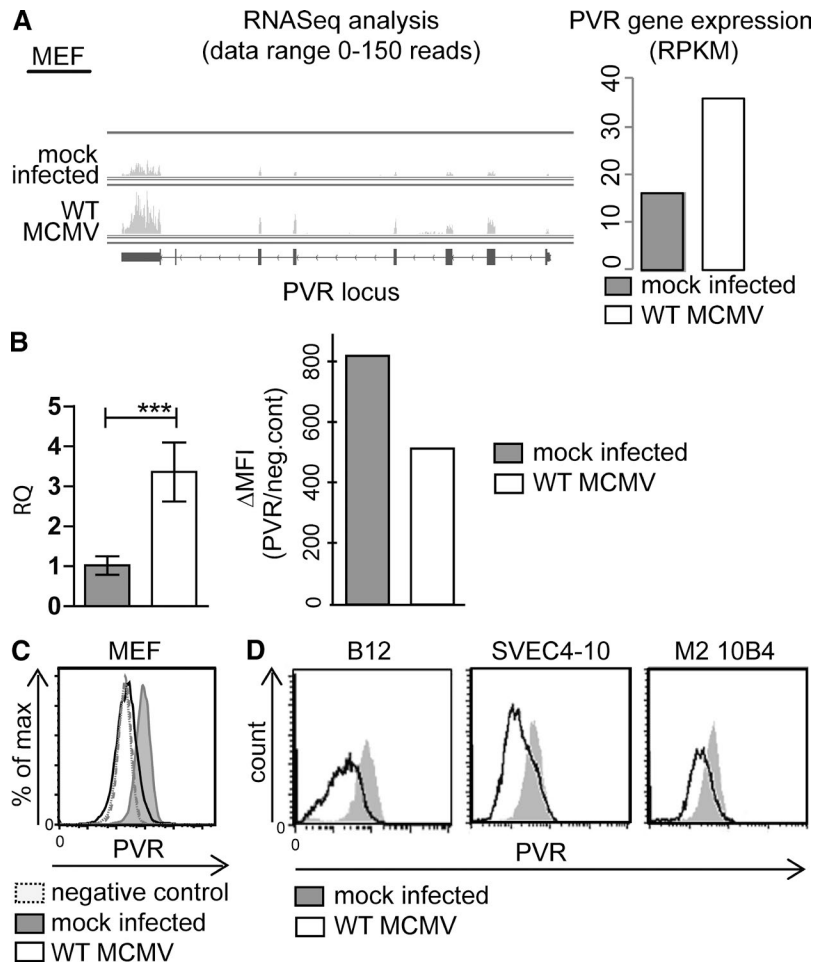


Figure 1. MCMV up-regulates PVR transcription but down-regulates its surface expression. (A) PVR locus with aligned reads from RNASeq analysis of infected and mock-infected MEF (Juranic Lisnic et al., 2013; left). Estimation of PVR gene expression by RPKM (reads per kilobase of exon model per million mapped reads; right). (B) Level of PVR transcript was measured in mock-infected BALB/c MEF and in WT MCMV-infected cells (left) that down-regulated PVR after 18 h p.i. (right) by quantitative RT-PCR. Data are representative from two independent experiments. ***, $P < 0.001$. (C) WT MCMV (1 PFU/cell, 16 h) or mock-infected MEFs were analyzed for the surface PVR by anti-PVR mAb or the isotype control. (D) Indicated cell lines, WT MCMV (3 PFU/cell, 16 h) or mock infected, were analyzed for the surface PVR expression. The analysis of surface PVR expression (C and D) was independently replicated six times.

was not feasible to construct individual deletion mutants for *m19* and *m21* ORFs. MEF was infected with indicated mutants and analyzed for PVR retention. Only the MCMV mutants lacking ORFs *m19* and *m20* ($\Delta m19/m20$) and ORFs *m20* and *m21* ($\Delta m20/m21$) were unable to retain PVR (Fig. 3 C). This result strongly suggested a role of *m20* in PVR regulation.

Our previous analysis of the transcriptional profile of the *m20* region (Juranic Lisnic et al., 2013) detected several overlapping transcripts. Consistent with these data, a RNA probe detected three dominant transcripts for the WT virus: transcripts of ~3, 2, and 1 kb (Fig. 3 D, left). Because in the available viral mutants, either all or none of those transcripts were missing (not depicted), three additional viral mutants were generated to identify the transcript involved in PVR regulation (Fig. 3 B, bottom). The transcriptional profile of the deletion mutant $\Delta m19.1$ and of WT MCMV was identical, whereas the $\Delta m20.0$ mutant gave no detectable transcripts (Fig. 3 D, right). The transcriptional profile of $\Delta m20.1$ virus showed a loss of the 2- and 3-kb transcripts, whereas the 1-kb transcript was preserved (Fig. 3 D). As can be seen from the Western blot analysis in Fig. 3 E, this $\Delta m20.1$ mutant was no longer able to retain the PVR.

To investigate whether the *m20* region corresponding to the 2- and 3-kb transcripts encodes the protein that regu-

lates PVR, we expressed fragments of the predicted *m20* protein as His-tagged proteins in *Escherichia coli* and used them as antigens to immunize mice. The newly generated mAb detected an MCMV protein in lysates of WT MCMV-infected cells but not in lysates derived from cells infected with the $\Delta m20.1$ mutant (Fig. 4 A). A dominant signal for an ~70-kD protein and a weak signal for a 55-kD form were observed. Endo H treatment of the 70 kD protein, named *m20.1*, increased the amount of its deglycosylated form of ~55 kD (Fig. 4 B). By immunoblotting PVR and *m20.1* in the lysates of MEF cells infected with WT MCMV or $\Delta m20.1$ mutant, we demonstrated that the retained PVR form is present only in cells infected with virus possessing an intact *m20.1* (Fig. 4 C). In parallel, we tested the expression of PVR on the surface of infected MEF cells by flow cytometry and showed that the virus lacking *m20.1* cannot down-regulate PVR (Fig. 4 D). In agreement with published work (Nabekura et al., 2014), the infection of primary DCs with WT MCMV resulted in the up-regulation of PVR expression. However, the PVR expression was still much lower than expression on the surface of cells infected with the virus lacking PVR inhibitor (Fig. 4 D). Finally, we confirmed that *m20.1* protein is required for PVR retention showing the protein-protein in-

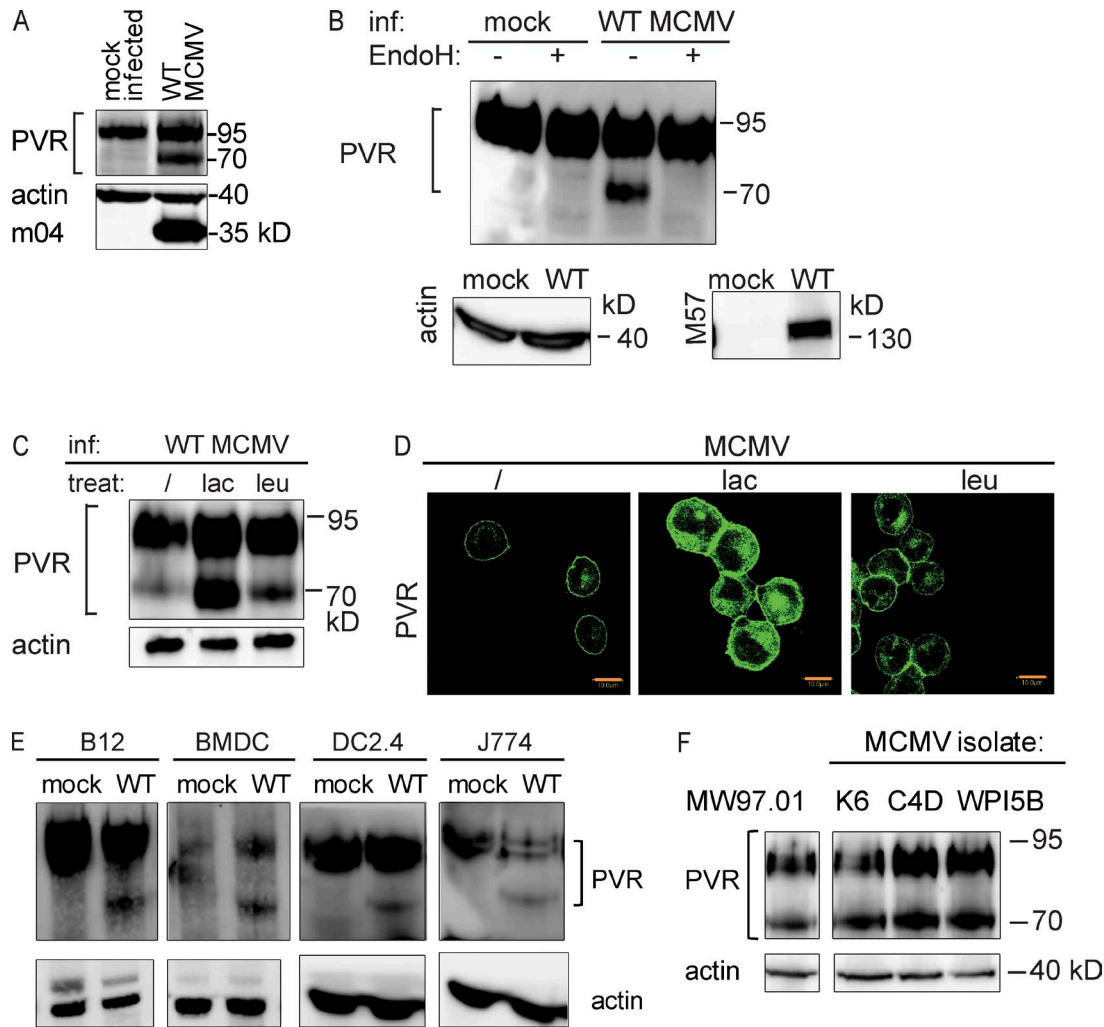


Figure 2. MCMV blocks PVR maturation in the ER and promotes its proteasomal degradation. (A) PVR was immunoblotted from WT MCMV (1 PFU/cell, 20 h) or mock infected MEF lysates with anti-PVR mAb; actin is shown as a loading control and MCMV m04 protein as a control of infection. Immunoblotting was independently replicated six times. (B) B12 cells were infected with WT MCMV (3 PFU/cell, 16 h) or mock infected. PVR was immunoblotted from EndoH-treated or untreated lysates (top); actin is shown as a loading control and MCMV M57 protein as a control of infection (bottom). (C) B12 cells were infected with WT MCMV (3 PFU/cell) and at 4 h p.i. treated with lactacystin (10 μ M), leupeptin (75 μ g/ μ l) or left untreated. 16 h p.i. lysates were analyzed with anti-PVR mAbs; actin is shown as a loading control. (D) B12 cells were infected with the virus lacking viral Fc receptor *m138*, treated as indicated for C and analyzed with anti-PVR mAbs, followed by FITC-labeled secondary Abs by IF. All images were equally adjusted using FluoView software; γ adjustment, 1.8; bar, 10 μ m. Experiments with lactacystin and leupeptin were independently replicated two times. (E) PVR was immunoblotted from mock or WT MCMV (3 PFU/cell, 20 h) infected lysates of indicated cells; actin is shown as a loading control. (F) PVR was immunoblotted from lysates of B12 cells infected with WT MCMV or three field isolates (2.5 PFU/cell, 16 h); actin is shown as a loading control. PVR immunoblotting (E and F) was repeated independently on each cell line at least two times.

teraction between the m20.1 and the ER retained PVR form of \sim 70 kD by coimmunoprecipitation (Fig. 4 E). Altogether, we have characterized the viral protein encoded in the *m20* gene region and proved that the ER-resident glycoprotein m20.1 is responsible for PVR retention.

In vivo attenuation of the MCMV mutant lacking the PVR inhibitor is partially mediated by NK cells

The next aim was to assess whether CMV regulation of PVR has functional relevance in vivo. BALB/c mice were

i.v. injected with Δ *m20.1* mutant or the respective control virus and viral titers in organs were determined 4 d p.i. (Fig. 5 A). The virus lacking the PVR inhibitor was strongly attenuated in vivo. Attenuation levels were similar in immunocompetent animals, as well as in SCID mice, suggesting a crucial role of innate immune cells in the control of the Δ *m20.1* virus (Fig. 5 B). These results indicate that viral regulation of PVR inhibits the early virus control in vivo. The infection of newborn mice, which are still immunologically immature and very sensitive to MCMV in-

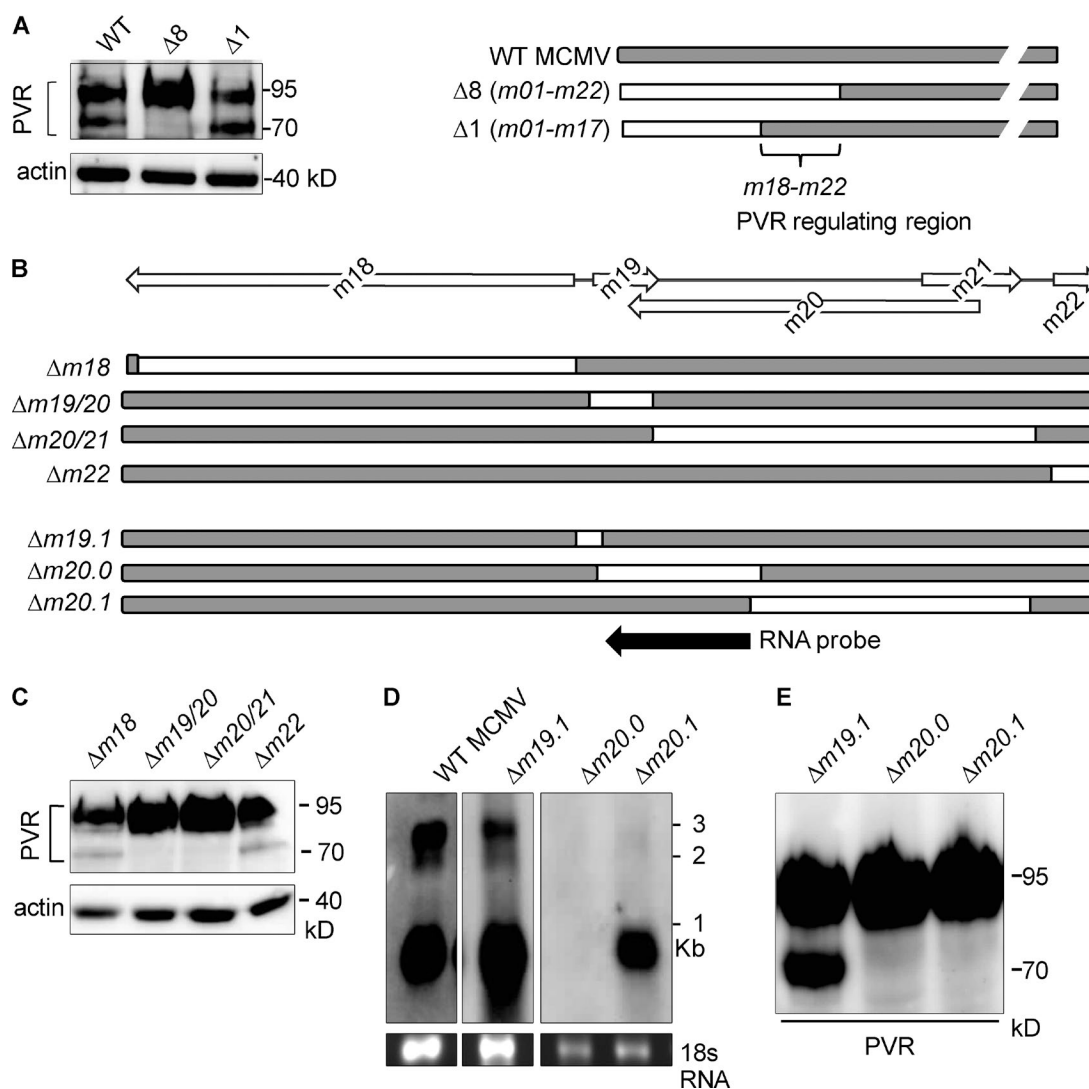


Figure 3. PVR is regulated by a gene located in the *m20* gene region. (A) B12 cells were infected with WT, $\Delta 8$ ($\Delta m01-m22$) or $\Delta 1$ ($\Delta m01-m17$) MCMV (3 PFU/cell) and immunoblotted with anti-PVR mAbs; actin is shown as a loading control. (A and B) White boxes represent deleted gene regions in recombinant viruses. (B) Previously annotated ORFs are shown as white arrows. The black arrow represents the RNA probe used for the Northern blot analysis in D. (C) PVR was immunoblotted from lysates of B12 cells infected with indicated mutants (3 PFU/cell, 16 h); actin is shown as a loading control. (D) MEF was infected with indicated viruses (0.3 PFU/cell, 48 h) or left uninfected. Transcripts were identified using the RNA probe (B). WT MCMV lane is from different gel, whereas $\Delta m19.1$, $\Delta m20.0$ and $\Delta m20.1$ are parts of the same gel analyzed with the same exposure. (E) MEF was infected with indicated viruses (1 PFU/cell, 20 h). PVR was immunoblotted from cell lysates. All experiments (A–E) were independently replicated at least two times.

fection, also results in attenuation of mutant virus in several tested organs (Fig. 5 C).

To assess whether the attenuated phenotype of the $\Delta m20.1$ mutant is the result of an enhanced sensitivity to NK cells, BALB/c and C57BL/6 mice were infected with either $\Delta m20.1$ or control virus. Several mice in each group were depleted of NK cells, and virus titers were determined on day 4 p.i. (Fig. 5 E). The results clearly demonstrated that NK cells contribute to efficient control of a virus lacking the PVR inhibitor, to varying extents depending on the tissue examined. These *in vivo* findings are in accordance with enhanced pro-

duction of IFN- γ by NK cells derived from $\Delta m20.1$ -infected mice compared with cells derived from mice infected with the control virus (Fig. 5 D). However, there were still significant differences between the titer of control virus and the $\Delta m20.1$ mutant after NK cell depletion, indicating that additional innate immune control mechanisms are involved (Fig. 5 E).

Dominant expression of DNAM-1 on inflammatory monocytes and macrophages after MCMV infection

The enhanced susceptibility of MCMV lacking the PVR inhibitor to immune control indicates a role of the DNAM-1–

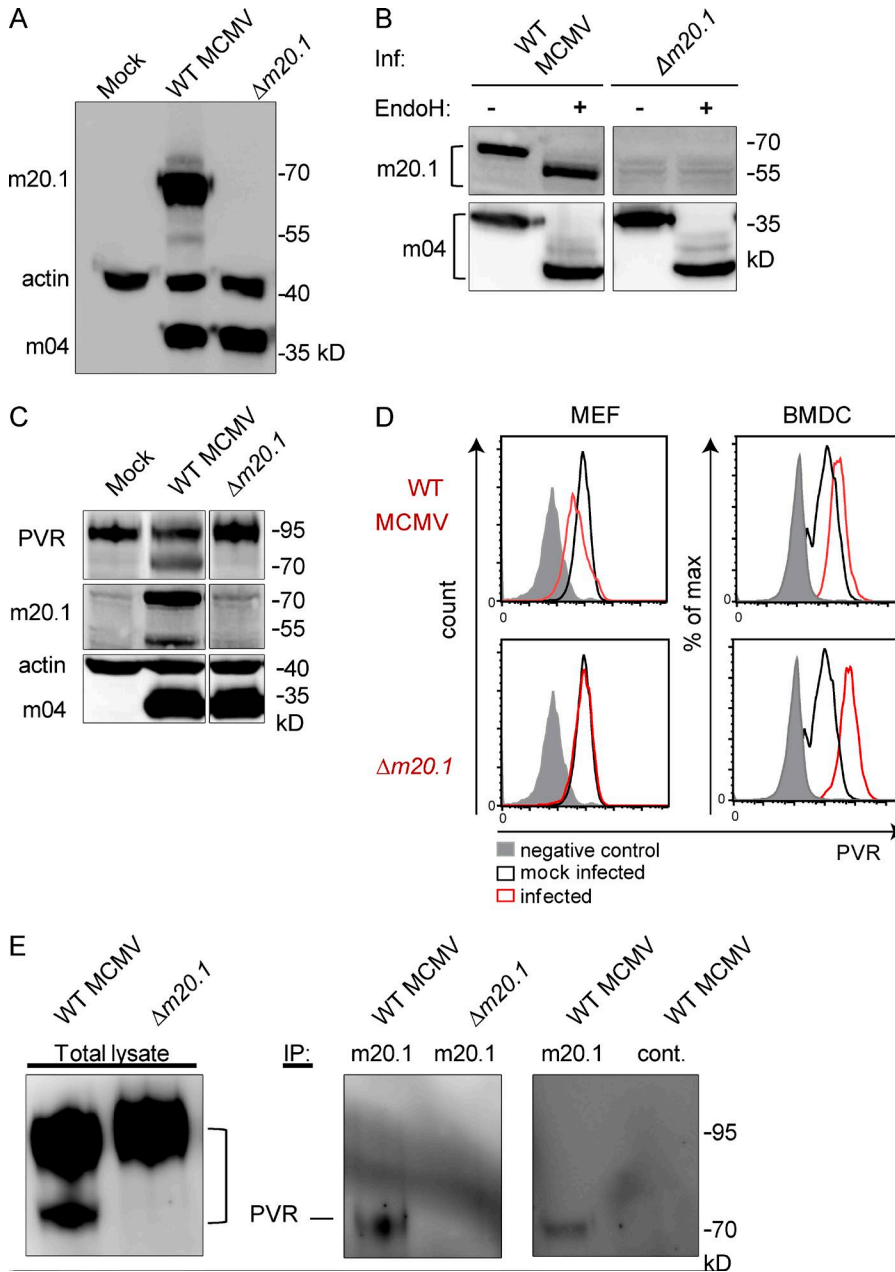


Figure 4. Characterization of the viral protein m20.1 that retains PVR. (A) m20.1 was immunoblotted from MCMV (indicated viruses, 1 PFU/cell, 20 h) or mock infected MEF lysates with anti-m20.1 mAb; actin is shown as a loading control and MCMV m04 protein as a control of infection. (B) MEF was infected with indicated viruses (0.8 PFU/cell; 20 h). MCMV m20.1 and m04 proteins were immunoblotted from EndoH-treated or untreated lysates with corresponding Abs; m04 is shown as a control of infection and EndoH treatment. (C) B12 cells were infected with indicated viruses (3 PFU/cell, 20 h) or mock infected. PVR and m20.1 proteins were immunoblotted from lysates with rat anti-mouse PVR mAb and anti-MCMV m20.1 mAb, respectively. Actin is shown as a loading control and m04 as a control of viral infection. Different parts of the same gel analyzed by same exposure are shown. (D) BMDCs or MEF cells were infected with either WT MCMV or $\Delta m20.1$ virus (3 or 1 PFU/cell, respectively). Infected and mock infected cells were analyzed for the surface PVR by anti-PVR mAb, or the isotype control, followed by anti-rat PE. (E) PVR was immunoblotted from MCMV-infected MEF lysates with anti-PVR mAb (left). The m20.1 was immunoprecipitated from the same lysates with the anti-m20.1 mAb or the control mAb, and PVR was subsequently immunoblotted from precipitates with anti-PVR mAb (right). All experiments (A–E) were independently replicated at least two times.

PVR pathway. Thus, we analyzed the expression of PVR receptors on NK cells and mononuclear phagocytes (Fig. 6, A–C). We found that the frequency of NK cells expressing DNAM-1 is higher in mice infected with MCMV, irrespective of the virus used. Yet the frequency of NK cells expressing TIGIT was also significantly higher in infected mice, whereas the frequency of CD96⁺ NK cells was significantly lower (Fig. 6 A). This fact might explain why NK cells fail to control $\Delta m20.1$ more efficiently. In contrast to NK cells, mononuclear phagocytes express almost no other PVR receptors except DNAM-1 (Fig. 6 B and not depicted). Moreover, we found that upon infection, the frequency of DNAM-1-expressing inflammatory monocytes (Fig. 6 B) and splenic macrophages

(Fig. 6 C) was dramatically increased. The surface density of DNAM-1 on all splenic macrophage subsets (including red pulp, marginal metallophilic, and marginal zone macrophages) was up-regulated upon infection (Fig. 6 C, bottom). Based on the pattern of expression of DNAM-1, TIGIT, and CD96 on mononuclear phagocytes, it appears to assume dominance of DNAM-1 in virus control.

Mice infected with $\Delta m20.1$ have higher level of proinflammatory cytokines in sera, as well as increased production of nitric oxide by inflammatory monocytes

Cytokine profiles in the sera of $\Delta m20.1$ -infected mice were in line with a more efficient antiviral activity (Fig. 6 D). Al-

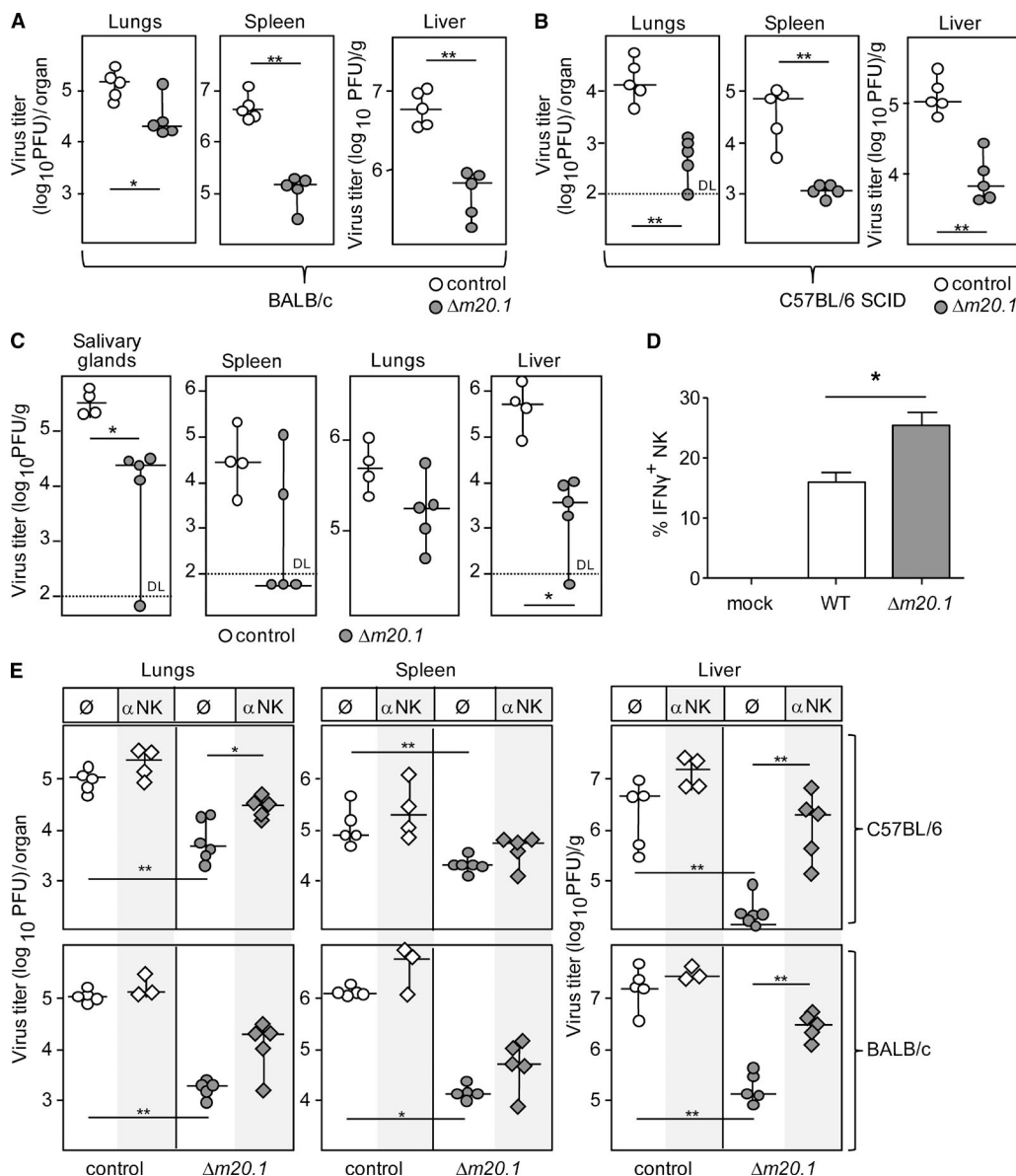


Figure 5. Heavy attenuation of MCMV lacking PVR inhibitor is caused in part by NK cells. BALB/c mice (A) or C57BL/6 SCID mice (B) were i.v. injected with 2×10^5 PFU/mouse (A) or 5×10^5 PFU/mouse (B) of $\Delta m20.1$ MCMV mutant generated on $\Delta m157$ background and $\Delta m157$ MCMV as a control virus. Titers in organs of individual mice 4 d p.i. are shown (circles); horizontal bars indicate the median values. (C) Newborn BALB/c mice were i.p. injected with 400 PFU/mouse of $\Delta m20.1$ MCMV mutant generated on $\Delta m157$ background or $\Delta m157$ MCMV. Titers in organs of individual mice 11 d p.i. are shown (circles); horizontal bars indicate the median values. Results from one of the three independent experiments (A) and one of the two independent experiments (B and C) are shown, with minimum four animals per group. DL, detection limit. *, $P < 0.05$; **, $P < 0.01$. (D) BALB/c mice were i.v. injected with 2×10^5 PFU of WT MCMV, $\Delta m20.1$ MCMV, or left uninfected. IFN- γ expression by splenic NK cells was determined by intracellular FACS analysis 1.5 d p.i. $n = 5$ animals; mean \pm SD; *, $P < 0.05$. Data are representative from three independent experiments. (E) C57BL/6 or BALB/c mice depleted for NK cells or undepleted were i.v. injected with 5×10^5 PFU/mouse or 2×10^5 PFU/mouse of $\Delta m157$ MCMV (control virus) or $\Delta m20.1$ mutant generated on $\Delta m157$ background. Titers in organs of individual mice 4 d p.i. are shown (circles); horizontal bars indicate the median values. Results from one of the two independent experiments with minimum three animals per group are shown. *, $P < 0.05$; **, $P < 0.01$.

though we were unable to define which subpopulations of mononuclear phagocytes are responsible for differences in cytokine production between WT- and $\Delta m20.1$ -infected mice, the sera of $\Delta m20.1$ -infected mice 1.5 d p.i. showed

increased level of IL-12p70, G-CSF, and IL-6, cytokines characteristic for the proinflammatory response of M1 mononuclear phagocytes (Martinez and Gordon, 2014). The level of IFN- γ , a major NK cell cytokine and an important activator

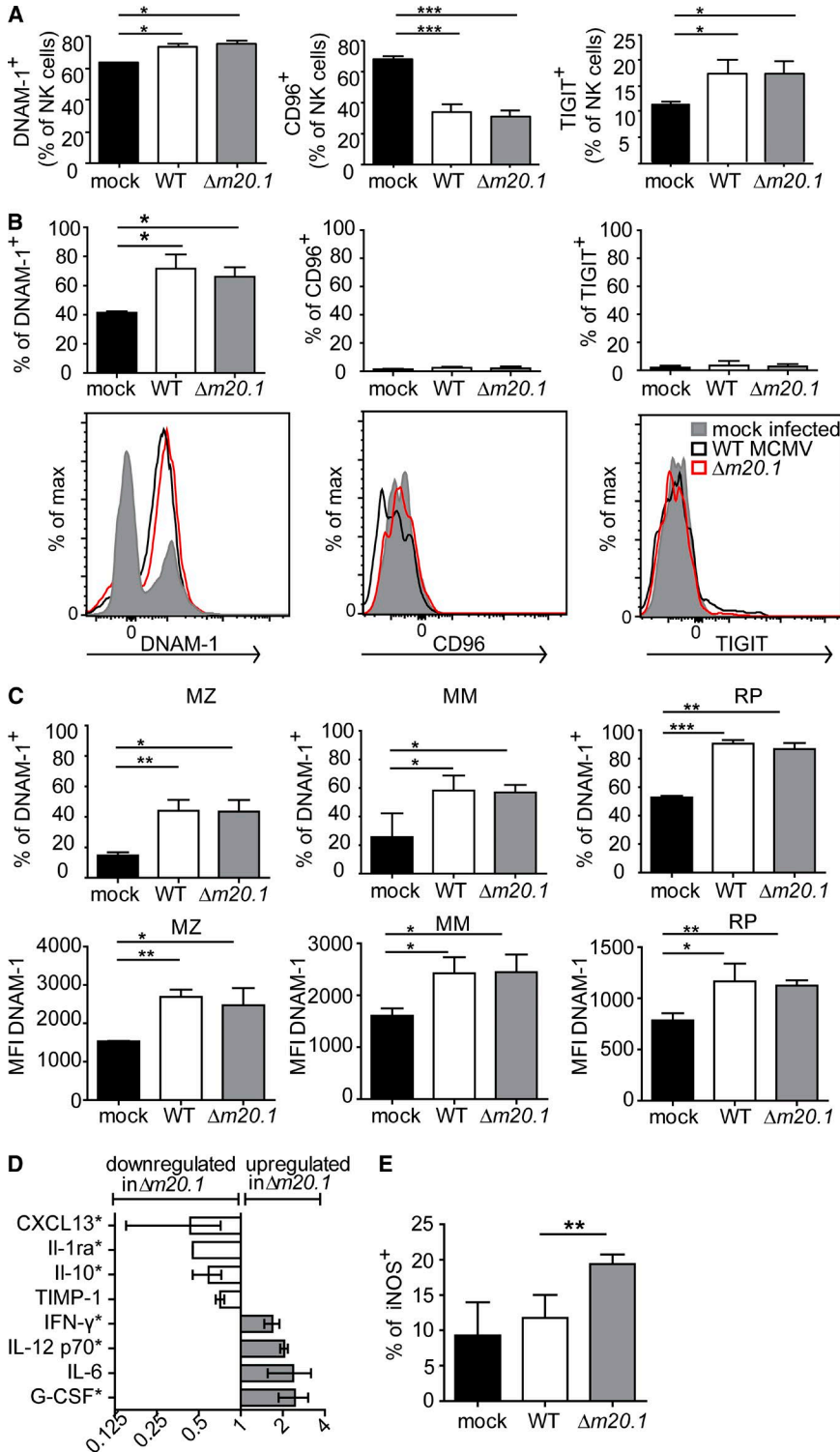


Figure 6. Macrophages and inflammatory monocytes in spleen selectively up-regulate PVR receptor DNAM-1 upon CMV infection. (A) BALB/c mice were i.v. injected with 2×10^5 PFU of WT MCMV, $\Delta m20.1$ MCMV, or left uninfected. 1.5 d p.i. percentages of DNAM-1⁺, CD96⁺, or TIGIT⁺ NK cells were determined from spleen by surface FACS analysis. (B) Mice were i.p. injected with 2×10^5 PFU of WT MCMV, $\Delta m20.1$ MCMV, or left uninfected and the expression of DNAM-1, CD96, and TIGIT determined on inflammatory monocytes by FACS analyses. (C) BALB/c mice were treated as described in B, and the expression of DNAM-1 on splenic macrophages determined by FACS analyses (RP, red pulp; MM, marginal metallophilic; MZ, marginal zone macrophages). (D) Dot blot analysis of cytokines, with capture antibodies spotted onto a membrane, in the sera of mice infected with WT MCMV or $\Delta m20.1$ MCMV. Two membranes with 40 different antibodies captured (in duplicate) were tested in each experiment with the sera of either WT MCMV or $\Delta m20.1$ MCMV infected mice. Experiment was repeated twice with different sera. Data selected for cytokines that were consistently up-regulated or down-regulated in both experiments and whose fold change was ≥ 2 in at least one of the experiments (*). Shown are mean values plus range. (E) BALB/c mice were treated as described in B. 1.5 d p.i. iNOS expression by inflammatory monocytes was determined by intracellular FACS analysis. (A and B [top] and C and E) $n = 5$ animals per group; mean + SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

of mononuclear phagocytes that is induced upon DNAM-1 signaling (de Andrade et al., 2014), was also elevated in the sera of mice infected with $\Delta m20.1$ (Fig. 6 D). Accordingly, the levels of IL-10, a hallmark of antiinflammatory response and inhibition of proinflammatory cytokine expression in

macrophages (Martinez and Gordon, 2014), were reduced in the sera of mice infected with $\Delta m20.1$ virus. The decrease was also observed for the CXCL13, IL-10–induced chemoattractant, and the antiinflammatory cytokine tissue inhibitor of metalloproteinases (TIMP-1). The level of another proin-

flammatory cytokine secreted by macrophages, IL-1, was not evidently different; however, $\Delta m20.1$ -infected mice had a lower amount of the IL-1 receptor antagonist (IL-1ra). Thus, the data indicate that m20.1 down-regulates the proinflammatory cytokine response via the PVR–DNAM-1 pathway.

Because cytokines induce iNOS to produce NO as an important effector mechanism of mononuclear phagocytes, we compared the production of iNOS by all major subsets of splenic mononuclear phagocytes, including red pulp macrophages, marginal metallophilic macrophages, and marginal zone macrophages, conventional DCs, plasmacytoid DCs, and inflammatory monocytes derived from mice infected with $\Delta m20.1$ or control MCMV (Fig. 6 E and not depicted). Inflammatory monocytes produced more iNOS after infection with $\Delta m20.1$ virus compared with WT MCMV (Fig. 6 E), suggesting their dominant role in observed phenotype in vivo.

DNAM-1- and iNOS-dependent attenuation of $\Delta m20.1$ by inflammatory monocytes

To assess the role of DNAM-1 in the early attenuation of $\Delta m20.1$, we used DNAM-1^{-/-} mice (Gilfillan et al., 2008). In mice lacking DNAM-1, the differences in virus titers between WT and $\Delta m20.1$ virus were reduced or abolished (Fig. 7 A). These findings are in accordance with the data obtained on iNOS production in DNAM-1^{-/-} mice (Fig. 7 B). Whereas in C57BL/6 mice the frequency of iNOS⁺ inflammatory monocytes was higher upon $\Delta m20.1$ infection compared with the WT MCMV infection, this difference was abolished in DNAM-1^{-/-} mice.

To further confirm the contribution of mononuclear phagocytes in the control of virus lacking PVR inhibitor, we treated infected mice with clodronate liposomes (Fig. 7 C). The treatment resulted in an increase of the WT virus titer, yet the increase of $\Delta m20.1$ virus titer was much more dramatic. Moreover, the treatment resulted in abolishment of the difference in titers between $\Delta m20.1$ and WT MCMV virus (Fig. 7 C). In agreement with the results shown in Fig. 5 E, the depletion of NK cells significantly affected the virus control, but the differences in virus titers between WT- and $\Delta m20.1$ MCMV-infected mice depleted of NK cells were still statistically significant. The simultaneous depletion of NK cells and mononuclear phagocytes by clodronate liposomes was necessary to abolish virus control in the spleen. These results indicate that, in addition to NK cells, mononuclear phagocytes play a role in attenuation of virus lacking PVR inhibitor.

To evaluate the impact of inflammatory monocytes that show higher level of iNOS expression in mice infected with $\Delta m20.1$ MCMV (Figs. 6 E and 7 B), we blocked CCL2, a chemokine required for exit of these cells from the BM and their recruitment to the inflamed tissue. Mice were treated with blocking anti-CCL2 antibodies, and virus titers were determined 4 d p.i. (Fig. 7 D). The blocking of CCL2 increased the titer of $\Delta m20.1$ and abolished the differences between viruses in spleen, whereas in liver the blocking effect

was partial. To further confirm that the differential levels of iNOS can explain the attenuation of $\Delta m20.1$ virus, we performed blocking of the iNOS–NO pathway by treating the mice with the inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME; Fig. 7 E). Our results demonstrate that blocking of iNOS production in vivo significantly increased the titer of the virus lacking PVR inhibitor but had no significant effect on the titer of WT MCMV virus.

DISCUSSION

While the outcome of viral down-regulation of cellular ligands for the activating receptors can be predicted, the situation is more complex for ligands such as PVR, which are recognized by both activating and inhibitory receptors (Martinet and Smyth, 2015). The outcome depends not only on the level of ligand down-regulation but also on the expression level and the affinity of its respective receptors. In this study, we have shown that the surface level of PVR is down-regulated by the MCMV protein m20.1, which affects the maturation of PVR in the ER, leading to its proteasomal degradation. The virus mutants lacking a PVR inhibitor are severely attenuated in vivo, indicating the dominance of the activating receptor DNAM-1 in deciding the outcome of the modulation of PVR levels. The early attenuation of mutant viruses lacking the PVR inhibitor was only partially dependent on NK cells, which can be explained by the fact that these cells induce both activating and inhibitory PVR receptors upon infection. However, the depletion of mononuclear phagocytes abolished the virus control, which correlates with dramatic up-regulation of DNAM-1 and absence of inhibitory PVR receptors on these cells even upon infection. In particular, we identified CCL2-dependent inflammatory monocytes as a major subpopulation controlling the virus lacking the PVR inhibitor via induction of iNOS.

Given that HCMV also retains PVR (Tomasec et al., 2005), one can assume that PVR regulation by this virus has a similar impact on the virus control as the one shown for the MCMV. Interestingly, in HCMV, the gene that regulates PVR, UL141, also inhibits the expression of another ligand of DNAM-1, nectin-2 (CD112; Prod'homme et al., 2010). In contrast, MCMV m20.1 solely regulates PVR, whereas another, thus far unidentified gene, is involved in the regulation of nectin-2 (unpublished data). Although HCMV and MCMV use different genes for regulation of PVR, the same functional outcome indicates the importance of DNAM-1 in virus control. It has been shown that inhibitory receptor TIGIT has a much stronger affinity for PVR than activating receptor DNAM-1 (Yu et al., 2009), and this might have functional consequences during CMV infection. Here, we demonstrated that MCMV infection partially reduces the PVR expression instead of its complete abolishment from the cell surface. This pattern is preserved even in cells that up-regulate PVR surface levels upon MCMV infection, such as DCs (this study and Nabekura et al., 2014). Thus, in all infected cells, the PVR expression after $\Delta m20.1$ MCMV infection exceeded the levels that were observed after WT MCMV infection. We hypoth-

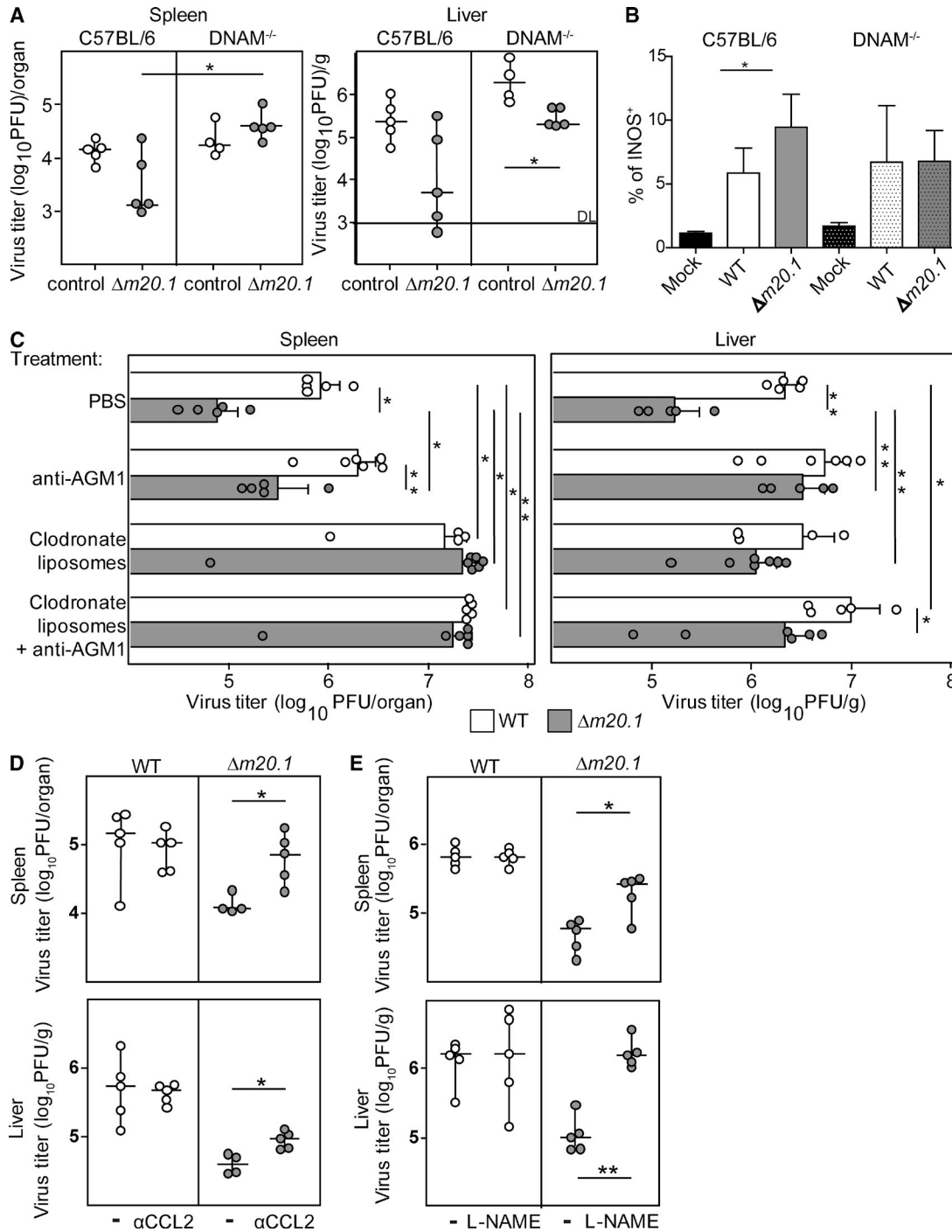


Figure 7. **DNAM-1- and iNOS-dependent control of $\Delta m20.1$ MCMV by inflammatory monocytes.** (A) C57BL/6 or DNAM^{-/-} mice were i.v. injected with 5×10^5 PFU of $\Delta m157$ MCMV (control virus) or $\Delta m20.1$ mutant generated on $\Delta m157$ background. Titers in organ of individual mice 4 d p.i. are shown (circles); horizontal bars indicate the median values; Results from one of the two independent experiments with minimum four animals per group are shown. *, $P < 0.05$. (B) C57BL/6 or DNAM^{-/-} mice were i.p. injected with 5×10^5 PFU of $\Delta m157$ MCMV (control virus), $\Delta m20.1$ mutant generated on $\Delta m157$ background, or left uninfected. 1.5 d p.i. iNOS expression by inflammatory monocytes was determined by intracellular FACS analysis. $n = 5$ animals; mean + SD; *, $P < 0.05$. (C) BALB/c mice were injected i.v. with 2×10^5 PFU of indicated viruses. For depletion of NK cells, mononuclear phagocytes or both subsets, groups of mice were treated with anti-AGM1, clodronate liposomes, or both, and virus titers were determined 4 d p.i. Group of mice injected with PBS was used as control. Results from one of the three independent experiments with minimum four animals per group are shown. Shown are mean values plus SEM. *, $P < 0.05$; **, $P < 0.01$. (D) BALB/c mice were injected i.p. with 2×10^5 PFU of indicated viruses. 1 d before infection and on the day of infection, in vivo blocking of CCL2 was performed by i.p. injection of the mAbs to CCL2. Titers in organs of individual mice 4 d p.i. are shown (circles). Results from

esize that MCMV might fine-tune the expression of PVR, to avoid recognition by the activating receptor while, at the same time, preserving ligation of the inhibitory receptor. In any case, this strategy could not protect the virus from control by mononuclear phagocytes, as we have shown that these cells express almost no inhibitory PVR-ligating receptors.

Previous studies have shown that inflammatory monocytes may play a dual role in antiviral responses because their functions can be beneficial or harmful, depending on the model and context of infection (Iijima et al., 2011; Lim et al., 2011; Daley-Bauer et al., 2012). In the context of CMV infection, monocytes have mainly been identified as cellular targets for viral dissemination and latency (Mitchell et al., 1996; Smith et al., 2004; Hargett and Shenk, 2010) or as modulators of antiviral immune response mediated by other immune cells (Hokeness et al., 2005; Daley-Bauer et al., 2012). Recently, it has been shown that patrolling, but not inflammatory, monocytes are involved in MCMV dissemination (Daley-Bauer et al., 2014), whereas inflammatory monocytes modulate adaptive immunity to MCMV (Daley-Bauer et al., 2012). However, less is known about possible direct antiviral effects of inflammatory monocytes in MCMV infection. Inflammatory monocytes use the same mechanisms as macrophages to control viruses, such as production of inflammatory cytokines or NO (Serbina et al., 2008). Indeed, NOS-deficient mice are more susceptible to MCMV infection (Noda et al., 2001). In line with this, our results show an increased iNOS production by inflammatory monocytes in mice infected with virus lacking the PVR inhibitor, which might explain more efficient control of this viral mutant. The role of inflammatory monocyte in iNOS-dependent control of MCMV was further confirmed by treatment of mice with the iNOS inhibitor L-NAME and by blocking of CCL2 chemokine. It is well established that inflammatory monocytes can give rise to tissue macrophages and DCs (Guilliams et al., 2014; Italiani and Boraschi, 2014). Both subsets have been shown to play a role in virus control and immunoregulation (Farrell et al., 2015; Gaya et al., 2015; Holzki et al., 2015). Thus, it is possible that these cells also contribute to enhanced control of the $\Delta m20.1$ virus. This particularly refers to macrophages, as our data show that all three splenic macrophage subsets selectively up-regulate DNAM-1 upon MCMV infection, whereas no such up-regulation was observed on DCs (unpublished data). Indeed, the results obtained after clodronate treatment support the role of other phagocytes.

Inflammatory cytokines promote the production of CCR2-binding chemokines and regulate monocyte/macrophage emigration from the BM, as well as their recruitment into the tissues in response to MCMV infection (Hokeness

et al., 2005; Crane et al., 2009; Wikstrom et al., 2014). In the sera of mice infected with the mutant lacking the PVR inhibitor, there was a shift toward proinflammatory cytokines, such as IL-12, which is crucial for IFN- γ production in the DNAM-1-driven response of NK cells (Magri et al., 2011). IL-12 has been shown to have a strong stimulating effect specifically on DNAM-1⁺ NK cells (Martinet et al., 2015). In line with this, our data show that IFN- γ , the major NK cell cytokine and an activator of macrophages, which is induced upon DNAM-1 signaling, was increased in the sera and in the splenic NK cells of mice infected with $\Delta m20.1$. Although it has been shown that pDCs are the main producers of IL-12 during early MCMV infection (Zucchini et al., 2008; Alexandre et al., 2014), alternative population of cells, such as CD11b⁺ DCs, can take over IL-12 production (Dalod et al., 2003). For example, CD14⁺ PBMCs are the main source of IL-12 during HCMV infection (Rölle et al., 2014). Moreover, one has to take into account the plasticity of the mononuclear phagocytes. On one hand, tissue macrophages polarize into M1, proinflammatory macrophages, and on the other hand, monocyte-derived macrophages and monocyte-derived DCs perform partially overlapping functions, including the secretion of proinflammatory cytokines (Italiani and Boraschi, 2014). In addition, monocytes can up-regulate CD11c without converting into DCs (Drutman et al., 2012). The discrimination of mononuclear phagocytes and their subpopulations is further complicated by the lack of selective markers (Gautier et al., 2012) and by the fact that MCMV infection or TLR signaling by itself changes the surface expression of several markers, leading frequently to their down-regulation as in the case of CD169, F4/80, CD11c, CD115, and others (Heise and Virgin, 1995; Singh-Jasuja et al., 2013; Daley-Bauer et al., 2014; Farrell et al., 2015). Knowing that most chemokines and cytokines can be produced by several cell types (Dalod and Biron, 2013), and that location, timing, and overall vigor of the immune response during CMV infection can affect their production, further studies are needed to determine the contribution of individual subsets to systemic cytokine levels in MCMV-infected animals.

Proinflammatory cytokines are also involved in immunopathology. HCMV is the most common cause of intrauterine viral infections and a major viral cause of neurological disease in children, including disorders of perceptual senses, such as hearing (Britt et al., 2013). A model of MCMV-induced hearing loss also points to a role of virus-induced inflammation (Bradford et al., 2015). Accordingly, antiinflammatory drugs can reduce such developmental abnormalities in MCMV-infected newborn mice (Kosmac et al., 2013). We assume that a tight con-

one of the two independent experiments with four to five animals per group are shown. *, $P < 0.05$. (E) BALB/c mice were injected i.v. with 2×10^5 PFU of indicated viruses. For blocking of NOS, mice were given sterile drinking water with or without L-NAME 3 d before infection and throughout the course of infection. Results from one of the two independent experiments with five animals per group are shown. *, $P < 0.05$; **, $P < 0.01$.

trol of activation of mononuclear phagocytes by PVR down-modulation might be beneficial for MCMV-infected newborn mice, particularly keeping in mind that immune mechanisms that are supposed to contain virus infection (e.g., NK cells and CD8 T cells) are not fully developed at this postnatal period. Therefore, the fact that the virus lacking PVR inhibitor is attenuated in neonatally infected mice can be a double-edged sword. Further studies are needed to assess the significance of viral regulation of PVR in reducing inflammation, histopathology, and neuronal abnormalities caused by cytokines and other soluble factors induced by the infection.

In conclusion, our data provide the strongest evidence so far for CMV control by mononuclear phagocytes and NK cells in which the DNAM-1–PVR pathway plays an essential role. In addition, the results demonstrate novel mechanism of viral regulation of paired receptors, which also differ in affinity to their common ligand. These results may be instrumental to identifying novel intervention targets and in designing novel vaccines and vaccine vectors. Our data also suggest that preservation of mononuclear phagocytes in individuals under immunodepletion regimens could reduce the risk of the primary viral infection and reactivation of latent viruses.

MATERIALS AND METHODS

Cells

SVEC4-10 (CRL-2181; ATCC), M2 10B4 (CRL1972; ATCC), J774A.1 (TIB67; ATCC), B12 (immortalized BALB/c fibroblasts), and MEF (mouse embryonic fibroblasts from BALB/c mice) were cultivated in Dulbecco's modified Eagle's medium supplemented with 10 or 3% fetal calf serum. DC2.4 cells (immortalized DCs) were cultivated in RPMI medium w/o mercaptoethanol. SP2/O (CRL 1581; ATCC) cells were cultured in supplemented or plain RPMI medium. To obtain BMDCs, BMDCs were cultured for 7 d in 10% RPMI complemented with GM-CSF.

Viruses

The bacterial artificial chromosome (BAC)-derived MCMV, MW97.01, has previously been shown to be biologically equivalent to the MCMV Smith strain (VR-194 [reaccessioned as VR-1399]; ATCC) and is here referred to as WT MCMV (Wagner et al., 1999). WT MCMV strains and various MCMV mutants used in these experiments were propagated on MEF, and virus stocks were prepared as described previously (Brune et al., 2001). MCMV mutants lacking different sets of genes or gene fragments were generated by site-directed mutagenesis on MCMV BAC, as described previously for $\Delta 8(m01-m22)$, $\Delta 1(m01-m17)$; Brune et al., 2006), $\Delta m138/fcr-1$ mutant (Crnković-Mertens et al., 1998), and $\Delta m157$ mutant (Bubić et al., 2004). The new MCMV mutants in region m18–m22 were generated on WT MCMV BAC. To avoid interference with NK cell activation via Ly49H receptor, a set of mutants in this region was also generated on the backbone of $\Delta m157$ BAC (Bubić et al., 2004). Primers used for generation of MCMV mutants of the *m18–m22* re-

gion on WT MCMV/C3X background are as follows: Primers for $\Delta m20.1$, 5'-M20_1-ACCTGGCCTATACTCACGTTGCCGTTGTGCAGGTCCGAGAACATGAGGACGACGACGACAAGTAA, 3'-M20_1-GTAAATGGACGGTTATTA AAAGATGAGGTCGTGTGACCTCTGTTACAGGAA CACTTAACGGCTGA; primers for $\Delta m20.0$, 5'-MP ACA CCCATCCCCTACCATTATGTTTCCCCGGTTCAT CTCGAGATCCTCAGAGTAAACTTGGTCTGACAG TTACC and 3'-MP TGGGCCAACGATCTGGCCGGA ATGTATCGCTGCGCCGTCTACTTCACCGCCGTG GACTCCAACGTCAAAGG; primers used for generation of MCMV mutants on $\Delta m157$ background, primers for $\Delta m18$ virus, 5'-M18-Kan TCGTCGTTAAGTATTTCTGCAAAG CATTGACGTCGTAATCGCTAACGACGCCAGTGT TACAACCAATTAACC and 3'-M18-Kan GACACT GGGCACGGTACCCGAACGAGAGGTTTCGAGGGTC GTCAGAGCGCCCGATTTATTCAACAAAGCCACG; primers used for $\Delta m19/m20$ virus, 5'-M19-Kan ATCATC GCCACACCCATCCCCTACCATTATGTTTCCCCG GTTCATCTCGAGCCAGTGTTACAACCAATTAACC and 3'-M19-Kan AGGCGAGTCTTCGGAGCTGTACGC TAGGGCGATCGCCATCACCTCTTCACGATTTA TTCAACAAAGCCACG; primers used for $\Delta m20/21$ virus, 5'-M20-Kan TGAAGAGGGTGATGGCGATCGCCCTAG CGTACAGCTCCGAAGACTCGCCTGCCAGTGTTA CAACCAATTAACC and 3'-M21-Kan GTCATGTTAAATG GACGGTTATTAAGATGAGGTCGTGTGACCTC TGTTACGATTTATTCAACAAAGCCACG; primers used for $\Delta m22$ virus, 5'-M22-Kan TAGCGCTCGATCGACGA GCGTCGGACAAAGAAACCCGGGAGAAGAAGGCCA GTGTTACAACCAATTAACC and 3'-M22-Kan TGATCG GATCGGACGGACCGGACCGGACCGCGACTGCTTG TCGGGCGGGTCCGATTTATTCAACAAAGCCACG; primers used for $\Delta m19.1$ (B84) virus, 5'-M19-MP-Kan AAG ACGCTCGTCTTATAACACCGACTGACGTTTACT CCGACTCAGGATGCCAGTGTTACAACCAATTAACC and 3'-M19-MP-Kan AAATCATACCATTTCGAGTCCG ATGTCCGTGTCTCACTTCTGGTTTCTTTGCGAT TTATTCAACAAAGCCACG; primers used for $\Delta m20.1$ (B85) virus, 5'-M20-MP-Kan ACCAACACCTGGCCTATACTC ACGTTGCCGTTGTGCAGGTCCGAGAACATGCCA GTGTTACAACCAATTAACC and 3'-M21-Kan GTCATG TAAATGGACGGTTATTAAGATGAGGTCGTGTGAC CTCTGTTACGATTTATTCAACAAAGCCACG.

Northern blot

Northern blot analysis was performed as described previously (Juranić Lisnić et al., 2013). In brief, RNA was isolated using the TRIzol reagent from mock or MCMV-infected MEF (0.3 PFU/cell). 1 μ g of RNA was separated, transferred to membrane, and cross-linked by UV irradiation. Membranes were incubated with DIG-labeled probes overnight at 67°C. Single-stranded RNA probe was generated by in vitro transcription from PCR products amplified with m19–m20 primers (Juranić Lisnić et al., 2013).

qPCR

BALB/c MEF was infected with 1 PFU of indicated viruses. The cells were collected with 2 mM EDTA, washed in PBS, and lysed in RLT buffer (QIAGEN). RNA was isolated using RNEasy Plus Mini kit according to manufacturer's (QIAGEN) instructions. RNA integrity was visualized on RNA Bleach gel (Aranda et al., 2012). RNA was treated with DNaseI (New England Biolabs), and then reverse transcribed using Protoscript II First Strand Synthesis kit (New England Biolabs) with random primers mix according to the manufacturer's protocol. PVR transcript was quantified in triplicates on Applied Biosystems' 7500 Instrument using TaqMan assay (Thermo Fisher Scientific) for mouse PRV (Mm00493398_m1) with GAPDH as endogenous control (Mm99999915_g1). No amplification from either PVR or GAPDH probes was observed in either RT controls or NTC samples. Relative quantitation was calculated using $\Delta\Delta CT$ method on 7500 Software V 2.0.5.

Protein expression and purification

Immunogens were subcloned into pQE-30 vector encoding N-6xHis tagged proteins, induced with 1 mM IPTG in BL21 DE3 cells, and purified under denaturing conditions using AKTA-prime. The pellet was lysed using 6 M guanidine hydrochloride, 20 mM sodium phosphate, and 500 mM sodium chloride; mixed in 1:1 ratio with the 8 M urea, 20 mM sodium phosphate, and 500 mM sodium chloride; and then applied to the Ni-NTA column. After elution (8 M urea, 20 mM sodium phosphate, and 500 mM sodium chloride, pH 4.0), the immunogen was diluted in 8 M urea, 50 mM sodium phosphate, 300 mM sodium chloride, and 10 mM imidazole (pH 7.4) and polished using Co-NTA column and the (8 M urea, 50 mM sodium phosphate, 300 mM sodium chloride, and 150 mM imidazole) elution conditions.

Generation of anti m20.1 mAbs

BALB/c mice were injected with 50 μ g of immunogen in complete Freund's adjuvant and, 2 wk later, in incomplete Freund's adjuvant. After 2 wk, the sera were screened for the antibody titer. The best responders were boosted with the immunogen in PBS. 3 d later, spleen cells were collected, and after lysis of red blood cells, fused with SP2/0 cells. The cells were seeded in 20% RPMI 1640 medium containing hypoxanthine, aminopterin, and thymidine for hybridoma selection and screened for mAbs using ELISA.

Flow cytometry

Uninfected or cells infected with indicated MCMV strains were stained for the surface PVR using rat anti-mouse PVR clone 3F1 (Hycult Biotech), followed by goat anti-rat IgG F(ab')₂-PE (sc-3829) or goat anti-rat IgG, F(ab')₂-FITC (sc-3825). Rat IgG2a isotype control (clone MEL.14) was generated and characterized in our laboratory.

Splenic leukocytes were prepared as previously described, and Fc receptors were blocked with 2.4G2 antibody

(Yokoyama and Kim, 2008). The following antibodies, purchased from eBioscience or BD, were used: CD3 ϵ (145-2C11), CD49b (DX5), NKp46 (29A1.4), TIGIT (GIGD7 and R&D cat.no. FAB7267A), CD96 (6A6 and 630612), DNAM-1 (10E5), IFN- γ (XMG1.2), CD11b (M1/70), CD11c (N418), CD19 (1D3), F4/80 (BM8), Ly6C (HK1.4), Ly6G (1A8-Ly6g), MERTK (DS5MMER), CD169 (3D6.112), SIGNR1 (cat.no. FAB1836P), CD64 (X54-5/7.1), MHCII (NIMR-4), PVR (TX56), B220 (RA3-6B2), PDCA-1 (eBio927), SA-EF710, and iNOS (CXNFT). For iNOS staining of myeloid cell subsets, splenocytes were incubated for 4 h in 10% RPMI with Brefeldin A and Monensin (Fig. S1). For IFN- γ staining, splenocytes were incubated as described for myeloid subsets, with addition of IL-2 (500 IU/ml). Subsequently, cells were surface stained, fixed, and permeabilized, followed by intracellular staining. Flow cytometry was performed by FACS Aria, FACSVerse, or FACScan (BD), and data were analyzed using FlowJo_v10 (Tree Star) software.

Immunofluorescence

B12 cells were infected with 3 PFU/per cell of recombinant MCMV lacking viral Fc receptor encoded by the *m138* gene. This virus was used to exclude the possibility of the rat anti-PVR mAb binding to the viral Fc receptor fcr-1. The equivalence of the WT MCMV and $\Delta m138$ virus with respect to PVR retention phenomenon was confirmed in a set of flow cytometry and Western blot analyses. Cells were supplemented with lactacystin (10 μ M) or leupeptin (75 μ g/ μ l) from the fourth h.p.i., and then fixed and analyzed for PVR after an additional 12 h. PVR was stained with the antibodies described in the Flow cytometry section, mounted using Mowiol mounting medium, and analyzed on RT with Olympus FV300 confocal laser scanning microscope using a PlanApo 60 \times NA1.4 oil objective (Olympus) and FluoView acquisition software.

Western blot analysis

Cell lysates were prepared using NP-40 lysis buffer (10 mM Sodium phosphate, pH 7.2, 150 mM sodium chloride, 2 mM EDTA, 1% NP-40, and protease inhibitors). Proteins, 75–100 μ g of lysate, were separated on 10–12% SDS-PAGE. For EndoH treatment, 75–100 μ g of lysate was incubated for 16 h with 25 mU of EndoH in 0.1 M phosphate buffer at 37°C. Lactacystin and leupeptin were used as described for Immunofluorescence. Membranes were incubated with anti-mouse PVR clone 3F1 (Hycult Biotech), anti-actin (EMD Millipore), anti-MCMV m04 (clone m04.10), anti-MCMV M57 (clone M57.01), and anti-MCMV m20.1 (clone m20.1.01; all generated in our laboratory). All samples were visualized using the UVITec imaging system.

Coimmunoprecipitation (CoIP)

MEF lysates were prepared as described for Western blot analysis and incubated overnight at 4°C under rotation with anti-m20.1 mAb, followed by 1-h incubation with protein

G-Sepharose beads (50 μ l; GE Healthcare). The precipitates were washed five times (1 ml each) with IP buffers before the samples were subjected to Western blot analysis. The membranes were incubated with anti-mouse PVR clone 3F1 (Hycult Biotech).

Cytokine detection

Mouse Cytokine Array Panel A Array kit (R&D Systems) was used according to the manufacturer's instructions. In brief, 80–100 μ l of sera previously mixed with antibody cocktail was added to the precoated membranes and incubated overnight. The membranes were incubated with streptavidin. The results were visualized by ImageQuant imaging system and analyzed with the ImageJ software (National Institutes of Health) with dot blot analysis plug-in.

Mice

BALB/c, C57BL/6, C57BL/6 SCID, and DNAM-1^{-/-} (on C57BL/6 background; Gilfillan et al., 2008) mice were housed and bred under specific pathogen-free conditions at the Central Animal Facility, Faculty of Medicine, University of Rijeka in accordance with the guidelines contained in the International Guiding Principles for Biomedical Research Involving Animals. The Ethics Committee at the University of Rijeka approved all animal experiments. Newborn mice and 8–12-wk-old mice were used.

Infection conditions, detection of MCMV, and depletion of cell subsets

Adult mice were injected either i.p. or i.v. with tissue culture-grown recombinant MCMV strains, at indicated doses, in a volume of 500 μ l of PBS. Organs were harvested at indicated time points, and virus titers were determined by a plaque-forming assay. In vivo depletion of NK cells was performed by i.p. injection of the mAbs to NK1.1 (PK136) or anti-AGM1 and of mononuclear phagocytes by i.p. injection of clodronate liposomes (200 μ l, 18 h before infection). In vivo blocking of CCL2 was performed by i.p. injection of the mAbs to CCL2 (clone 2H5; BioXCell; 200 μ g/mouse), 1 d before infection and on the day of infection. Newborn BALB/c mice were injected i.p. with 400 PFU of either $\Delta m20.1$ MCMV mutant generated on $\Delta m157$ background or $\Delta m157$ MCMV as a control virus in a volume of 50 μ l of PBS. Organs were harvested 11 d p.i., and virus titers were determined by a plaque-forming assay. In vivo inhibition of NOS: L-NAME (Sigma-Aldrich) was administered ad libitum in drinking water (5 mM) starting 3 d before infection. L-NAME solutions were changed daily. Control groups received only drinking water.

Statistical analysis

Statistical significance of the differences between experimental groups of animals in viral titers was determined by the two-tailed Mann-Whitney *U* test and for surface protein expression or intracellular iNOS and IFN- γ detection on different cell subsets by unpaired, two-tailed Student's *t* test.

Online supplemental material

Fig. S1 shows gating strategies for mononuclear phagocytes. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20151899/DC1>.

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