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 DNA 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 (Reddedge, 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 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; 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 TIG IT, 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 (Chadéan et al., 1994; Groemeier et al., 2000; Mason 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 upon MCMV infection (Nabekura et al., 2014; Smith et al., 2008) showed the same PVR retention phenotype. 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. 2E, 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. 2F). Thus, PVR retention is conserved among 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 (Δ8 virus, Fig. 3A). Because PVR maturation in cells infected with an MCMV mutant lacking the segment of first 17 genes (Δ1 virus) was comparable with the WT MCMV, we concluded that the PVR regulator must lie in the m18-m22 gene region (Fig. 3A). 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. 3B, top). Because ORF m20 significantly overlaps with ORFs m19 and m21, it...
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 (Δm19/m20) and ORFs m20 and m21 (Δm20/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 Δm19.1 and of WT MCMV was identical, whereas the Δm20.0 mutant gave no detectable transcripts (Fig. 3 D, right). The transcriptional profile of Δm20.1 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 Δm20.1 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 regulates 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 Δm20.1 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 Δm20.1 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-

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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.
interaction between the m20.1 and the ER retained PVR form of ∼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 Δm20.1 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 Δm20.1 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-
fection, also results in attenuation of mutant virus in several tested organs (Fig. 5 C).

To assess whether the attenuated phenotype of the Δm20.1 mutant is the result of an enhanced sensitivity to NK cells, BALB/c and C57BL/6 mice were infected with either Δm20.1 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 production of IFN-γ by NK cells derived from Δm20.1-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 Δm20.1 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-
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 Δm20.1 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 Δm20.1 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 Δm20.1-infected mice were in line with a more efficient antiviral activity (Fig. 6 D).
though we were unable to define which subpopulations of mononuclear phagocytes are responsible for differences in cytokine production between WT- and Δm20.1-infected mice, the sera of Δm20.1-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 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 × 10⁵ PFU/mouse (A) or 5 × 10⁵ PFU/mouse (B) of Δm20.1 MCMV mutant generated on Δm157 background and Δm157 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 Δm20.1 MCMV mutant generated on Δm157 background or Δm157 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 × 10⁵ PFU of WT MCMV, Δm20.1 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 ± 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 × 10⁵ PFU/mouse or 2 × 10⁵ PFU/mouse of Δm157 MCMV (control virus) or Δm20.1 mutant generated on Δm157 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.
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 Δm20.1 (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 Δm20.1 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 proinflam-
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 metallophagocytic 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 infection (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 subgroup 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 TIG IT 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.
Figure 7. **DNAM-1– and iNOS–dependent control of Δm20.1 MCMV by inflammatory monocytes.** (A) C57BL/6 or DNAM−/− mice were i.v. injected with 5 × 10^5 PFU of Δm157 MCMV (control virus) or Δm20.1 mutant generated on Δm157 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 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 Δm157 MCMV (control virus), Δm20.1 mutant generated on Δm157 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 to control viruses, such as production of inflammatory cytokines or NO (Hokeness et al., 2005; Crane et al., 2009; Wikstrom et al., 2014). 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 confirmed by treatment of mice with the iNOS 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 Δm20.1. 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, CD11b, 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-
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 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 BAL-B/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 differing sets of genes or gene fragments were generated by site-directed mutagenesis on MCMV BAC, as described previously for Δm18–m22, Δ11(m01-m17; Brune et al., 2006), Δm138/Δir–1 mutant (Crnković-Mertens et al., 1998), and Δm157 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 Δm157 BAC (Bubić et al., 2004). Primers used for generation of MCMV mutants of the m18–m22 region on WT MCMV/C3X background are as follows: Primers for Δm20.1, 5′–Δm20.1-ACCCTGCCCTATACTCAAGTTG CGCGTTGTGCAGGTGTCGGAGAACATGAAGGACGACG ACGACAGTAGA, 3′–Δm20.1-GTAATTGAGCTGGTTGAC TTCTGAGTTTCTGTTACAGGAA CACTTACAGCTGTA; primers used for Δm20.0, 5′–MP ACA CCCATCCCTCATTATGGTTCTCCGGTTCAT CTTCGAATCTCCAGGATAAACTTGGTGTACAGG TTACC and 3′–MP TGGGCCAACGATCTGGCGCGA ATGTATCCTGCGCCGTCTACTTCCAGGCG TGGACTCCAGTCAAAG; primers used for generation of MCMV mutants on Δm157 background, primers for Δm18 virus, 5′–M18-Kan TCGTCTTTAGATTTCTGGCAAAG CATTCCGACGTCTGATATCGTAAACGCGCAGGAGCTT ATACACAAATCACC and 3′–M18-Kan GACACG GCCAGCTACCCGAACGAGAGGGTCGAGGTC GTCCAGGCGCCGTTATTATCAAAAGCCAGC; primers used for Δm19/m20 virus, 5′–M19-Kan ATCATE GCAACACCATCTCATTATGTTTTCCCGC GTTCACTCTCAGGACTGTTACCAATTAC and 3′–M19-Kan AGGCGATCTCCGGACGCGTACTGGC TAGGGCGATGCACACCTCCTACTGAGTTA TTTCAACAAAGCCAGC; primers used for Δm20/21 virus, 5′–Δm20–Kan TGAAGAGGTTGTTAGGCTGAC TCCGACTCCAGTCCGAGACTCCTGCCGAGTTA CAAACTTTAAC and 3′–Δm21–Kan GTATGTTAAATG GACGGTTTTAAAAAGATAGGTCGCGTGTGACCTC TGTTACGTTTATCCAAAGCCAGC; primers used for Δm22 virus, 5′–M22–Kan TAGCCGCTCTGATCGACGA GGGTCGGAACAAAG AACCCGGAGAAGAGCCA GTTATACAAACCTAACCC and 3′–M22–Kan TAGATCG GATCGGACGGACCGGACGGACGGCAGCTGTTGT TCGGGCGGTGCGTTATTTTACAACAAAGCCAGC; primers used for Δm19.1(B84) virus, 5′–M19′-MP–Kan AAAG CCGCTCTGTTTTAATAACACCGAGCTGAGTTTACT CCGACTCCAGTGGCTGTTGACATCAACAAATTACC and 3′–M19′-MP–Kan AAATCATACATTCCAGGTCCG ATCTCCCGTCTCCTACTTGTCTTGTCCGAT TTATCTCAAAAGCCAGC; primers used for Δm20.1(B85) virus, 5′–M20′-MP–Kan ACCAACACCTGGCTTATCTACT CAGTTGGCCGCTTTGTCGAGCTGAAATCAGCCA GTGTTACAAACCTAACCC and 3′–M21–Kan GTATG TAAATGGAAGGTTTTAAAAGATAGGTCGCGTGTGAC CTCTGTTACGTTTATCCAAAGCCAGC.

**Northern blot**

Northern blot analysis was performed as described previously (Juranic Liscic et al., 2013). In brief, RNA was isolated using the Trizol reagent from mock or MCMV-infected MEF (0.3 PFU/cell). 1 μg 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 (Juranic Liscic 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 PVR (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 ΔΔCT 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 lyzed 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 µg 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, aminopterine, 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: CD3ε (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 (DSSMMEK), CD169 (3D6.112), SIGNR1 (cat.no. FAB1836P), CD64 (X5-5/7.1), MHCIIN (NIRM-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 FACSARia, FACSVersus, 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 Δm138 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 µM) or leupeptin (75 µg/µl) 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× 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 µg of lystate, were separated on 10–12% SDS-PAGE. For EndoH treatment, 75–100 µg of lystate was incubated for 16 h with 25 µM 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 m20.1 (clone m20.1.01; all generated in our laboratory). All samples were visualized using the UVITec imaging system.

Comunoprecipitation (CoP)
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

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G–Sepharose beads (50 µl; 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 µl 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 Center for Molecular 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 µl of PBS. Organs were harvested at indicated time points, and virus titers were determined by 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 µl, 18 h before infection). In vivo blocking of CCL2 was performed by i.p. injection of the mAbs to CCL2 (clone 2H5; BioXCell; 200 µg/mouse), 1 d before infection and on the day of infection. Newborn BALB/c mice were injected i.p. with 400 PFU of either Δm20.1 MCMV mutant generated on B/c mice were injected i.p. with 400 PFU of either Δm157 background or Δm157 MCMV as a control virus in a volume of 50 µl of PBS. Organs were harvested 11 d p.i., and virus titers were determined by plaque-forming assay. In vivo inhibition of NOS: t–NAME (Sigma-Aldrich) was administered ad libitum in drinking water (5 mM) starting 3 d before infection. t–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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