Title
Cytomegalovirus immunoevasin reveals the physiological role of "missing self" recognition in natural killer cell dependent virus control in vivo.

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A balance of activating and inhibitory signals, mediated through distinct classes of receptors found on their surface, regulates NK cell activity (Ortaldo and Young, 2005; Lanier, 2008). Activating signals are interrupted when inhibitory receptors on NK cells engage MHC class I (MHC I) molecules on target cells. This dual receptor system, also known as the "missing self" activation mechanism (Kärre et al., 1986), enables NK cells to detect virally infected cells that exhibit a reduced expression of MHC I molecules. To compromise antigen presentation in the context of MHC I molecules, and thus evade the attack by CD8+ T cells, cytomegaloviruses (CMVs) encode immunoevasin proteins that can downmodulate the expression of MHC I on the cell surface (Hengel et al., 1999), most likely by a recently described mechanism demonstrating that the down-regulation of MHC I from the surface of infected cells is a result of MHC I turnover and inhibited supply of newly synthesized molecules, rather than the active downmodulation of the surface-resident portion (Lemmermann et al., 2010). However, this lowers inhibitory interactions and triggering through inhibitory Ly49 receptors, and might activate NK cells through a missing self–dependent mechanism. In addition, CMVs actively compromise NK cell activation using other strategies, such as the
downmodulation of cellular ligands for activating NK cell receptors (e.g., NKG2D) or miRNAs (Jonjić et al., 2008). Because of an efficient and redundant viral immunoevasion from NK cells, most laboratory mouse strains, as well as feral mice, fail to mount a significant NK cell response toward mouse CMV (MCMV; Scalzo et al., 2005), with the exception of a few mouse strains that possess an activating Ly49 receptor, which specifically recognizes infected cells and therefore circumvents viral immunoevasion (Arase et al., 2002; Smith et al., 2002; Adam et al., 2006; Kielczewska et al., 2009).

The aim of this study was to explain the mechanisms by which MCMV avoids NK cell control, in spite of the down-regulation of MHC I molecules on the surface of infected cells. As shown previously, MCMV encodes three proteins involved in the regulation of MHC I expression (Wagner et al., 2002; Holtappels et al., 2006; Pinto et al., 2006). The m152-encoded gp40 glycoprotein arrests MHC I at the level of the ERGIC/cis–Golgi compartment (Ziegler et al., 1997), whereas m06-encoded gp48 redirects MHC I complexes to lysosomes for degradation (Retusch et al., 1999). The third MCMV regulator of MHC I expression is gp34, encoded by the m04 gene (Kleijnen et al., 1997). Unlike the other two, m04/gp34 does not prevent the surface expression of MHC I, but instead binds to these proteins in the ER, forming complexes that can reach the cell surface (Kleijnen et al., 1997). Although it has been proposed that m04/gp34 cooperates with two other immunoevasins to compromise antigen presentation to CD8+ T cells (Kavanaugh et al., 2001a), recently published data indicate that m04 is not a CD8+ T cell immunoevasin on its own, but rather an antagonist of m152 function (Holtappels et al., 2006; Pinto et al., 2006). It has been suggested that m04/gp34 might inhibit NK cell activation through its ability to escort MHC I to the cell surface to engage inhibitory NK cell receptors (Kleijnen et al., 1997). We have recently shown that m04/gp34 is essential for the recognition of infected cells by the activating Ly49P receptor, which recognizes H-2D^d together with m04 product and another, thus far unidentified viral component (Kielczewska et al., 2009).

Here, we provide evidence that MCMV lacking the m04 gene (Δm04) is attenuated in an NK cell– and MHC I–dependent manner in several mouse strains. By using reporter cells expressing the inhibitory receptor Ly49A, we have shown that the expression of m04 in infected cells allows the engagement of this receptor by enabling MHC I molecules to reach the cell surface, despite the two viral MHC I inhibitors. Our studies provide the first evidence that NK cell recognition of the missing self is relevant in the recognition and control of a viral pathogen in vivo.

**RESULTS**

Δm04 MCMV is attenuated in vivo in an NK cell– and MHC I–dependent manner

Based on the ability of m04 to escort MHC I molecules to the surface of infected cells, we have hypothesized that its primary function is blocking NK cell activation via the missing self mechanism. If this is correct, the deletion of m04 should attenuate MCMV in an NK cell–dependent manner by decreasing the expression of ligands for inhibitory NK cell receptors. To test this, we infected BALB/c mice with either WT MCMV or virus lacking m04 (Δm04). A portion of mice in each group was depleted of NK cells, and the virus titer in the spleen was determined 3 d post infection (dpi). We have observed a significantly lower viral replication of the Δm04 mutant, which was reverted to WT MCMV titer after depletion of NK cells (Fig. 1 A). These results clearly support the initial hypothesis that m04/gp34 causes NK cell inhibition. We have also tested whether this phenotype is dependent on the expression of MHC I molecules. The aforementioned difference in viral titers between Δm04 and WT MCMV was absent in TAP1-deficient mice (Tap1^−/−) and mice deficient for β2 microglobulin (β2m^−/−), both on BALB/c background (Fig. 1 B).

Moreover, we have tested the impact of m04 in several other mouse strains (Fig. 1 C). Even more pronounced than in BALB/c mice, the absence of m04 resulted in an NK cell–dependent viral attenuation in C3H/J (H-2^k), CBA/J (H-2^k), BALB.K (H-2^k), and B10.M x BALB/c (BALB.F; H-2^k) mouse strains. However, in C57BL/6 (H-2^b) mice no difference in viral titers was observed between Δm04 and WT MCMV (Fig. 1 D). To avoid a strong activation of C57BL/6 NK cells through the Ly49H receptor, which can abolish the impact of m04, we have used BALB.B mice, of the identical H-2 haplotype but different NKC background. As expected, without Ly49H, BALB.B mice showed a high virus titer; however, no attenuation with Δm04 observed (Fig. 1 D). Therefore, we concluded that the attenuation of Δm04 mutant is NK cell– and MHC I–dependent. This phenomenon is clearly seen in mice of the H-2^k and, to a lesser extent, the H-2^b backgrounds, but is most likely completely absent in H-2^d animals.

MCMV m04 protects infected cells from NK cell–mediated killing

To further elaborate the role of m04 in protecting infected cell from NK cell–mediated recognition, we have tested ex vivo the ability of splenic cells from naive BALB/c and C3H/J mice to lyse B12 (SV40-transformed BALB/c fibroblasts; H-2^d) and SVEC4-10 (C3H/J-derived SV40 transformed endothelial cells; H-2^d) cells, infected with WT or Δm04 MCMV or left uninfected. WT infected B12 and SVEC4-10 cells were both lysed less efficiently as compared with noninfected targets. However, Δm04-infected targets were more susceptible to NK cell lysis than WT-infected cells (Fig. 2 A).

Although it is difficult to determine if viral titers in the spleen are always correlated with the ex vivo function of NK cells, it has to be pointed out that the contribution of m04 to the concealment of infected cells in vitro is much less pronounced. Even though in vivo MCMV can infect many different cell types, the antiviral activity of NK cells differs depending on the affected organ or tissue. Therefore, it is not completely unexpected that the extent of cytolytic susceptibility...
of certain cell lines does not directly correlate with NK cell efficacy in vivo.

Because both B12 and SVEC4-10 target cells constitutively express NKG2D ligands (Fig. 2 B), uninfected cells are likely lysed by NK cells via an NKG2D-dependent mechanism, but the role of other activating receptors cannot be excluded. It is notable that MCMV down-regulates ligands for NKG2D receptor on these cells, with the exception of surface-resident RAE-1β in SVEC4-10 cells, which is constitutively resistant to MCMV (Arapovic et al., 2009). Because m04 does not interfere with the expression of these ligands (Fig. 2 B), the decrease in NK cell lysis of WT MCMV–infected cells as compared with uninfected targets might be caused by a decreased signal from an activating receptor (e.g., NKG2D) and/or NK cell inhibition provided by m04–MHC I complexes. Regardless, in the absence of m04, MCMV infected cells regain susceptibility to NK killing. Together with in vivo results, this finding confirms the inhibitory role of m04 in NK cell–mediated virus control.

We have also performed the same assay on the macrophage cell line RAW264.7 derived from BALB/c mice. Contrary to B12 and SVEC4-10 cells, WT MCMV–infected RAW264.7 cells were lysed to a greater extent than noninfected targets, whereas Δm04-infected targets were again more susceptible to lysis than WT-infected cells (Fig. S1).

Figure 1. Δm04 is attenuated in vivo in an NK cell– and MHC I–dependent manner. (A–D) Indicated strains of mice were injected i.v. with 2 × 10⁵ PFU (except C57BL/6, which received a dose of 3 × 10⁵ PFU) of WT or Δm04 MCMV and 3 dpi viral titers in spleens were assessed. Where indicated, mice were depleted of NK cells by injection of 20 µl of anti-AGM1. P values, unpaired two-tailed Mann-Whitney test. A circle depicts the titer for each individual mouse; a small horizontal line indicates the mean. Data are representative of at least two independent experiments with four to five mice per group.
decreased stimulatory potential and therefore a less dramatic impact of m04 absence, which is in agreement with the results obtained in vivo (Fig. 3 A, middle). As a control, Ly49P-expressing reporter cells were not activated after coculture with uninfected or Δm04-infected cells (Fig. 3 A, left), which further supports our previous results (Kielczewska et al., 2009).

Because Δm04 was not attenuated in C57BL/6 or in BALB.B mice, we tested the ability of H-2^b cells, either infected or uninfected, to engage the Ly49A receptor. As shown in Fig. 3 A (right), in contrast to Ly49A^CD3^ reporter cells cocultured with CBA/J or BALB/c MEFs, MEFs derived from C57BL/6 mice failed to activate reporter cells, confirming that Ly49A does not bind to MHC I molecules of the H-2^b haplotype (Karlhofer et al., 1992), and again supporting our in vivo finding. Consequently, the infection of C57BL/6 cells with either WT or Δm04 MCMV did not result in the activation of Ly49A^CD3^ reporter cells. Altogether, these findings demonstrate that m04 plays an essential role in preventing NK cell activation via the missing self mechanism by providing ligands for inhibitory NK cell receptors, at least in the context of H-2^d^ and H-2^k^ haplotype.

A consistent increase in GFP expression by Ly49A reporter cells cocultured with WT MCMV-infected cells made us conclude that association of m04/gp34 with MHC I molecules increases their binding to Ly49A. To test this assumption, we constructed a reporter cell line expressing the Ly49A ectodomain fused to the stalk, transmembrane, and cytoplasmic domains of Ly49H, which associates with DAP12 adaptor protein containing one ITAM motif (Ly49A^HCTS^) contrary to Ly49A-CD3^ chimera (Ly49A^CD3^), which by itself encodes three ITAM motifs. This Ly49A^HCTS^ chimeric receptor shows less binding to MHC I molecules and generates a weaker signal (Ito et al., 2009). Therefore, Ly49A^HCTS^ reporter cells were poorly activated by uninfected or m04 MCMV-infected cells (of H-2^k^ or H-2^d^ haplotype), but were still strongly activated when co-cultured with WT MCMV-infected cells (Fig. 3 B). Similar results were obtained with reporter cells expressing the Ly49A allele from C57BL/6 mice (Fig. 3 B), which also indicated that the lack of interaction between Ly49A and MHC I in C57BL/6 mice is determined by MHC I and not by the Ly49A allele. Therefore, we concluded that m04–MHC I complex might have a higher affinity for Ly49A as compared with native MHC I.

To assess whether the effect of m04 is restricted to Ly49A, we generated 2B4 NFAT-GFP reporter cells expressing Ly49C. Ly49C is another inhibitory receptor with high affinity for H-2^K^, H-2^D^, H-2^K^, H-2^D^, and H-2^D^; Ly49C is found in most NK haplotypes (including BALB/c, CBA/J, and C57BL/6; Brennan et al., 1996a). However, whereas Ly49A is present on ~5–10% of NK cells, Ly49C is expressed on ~50% of NK cells in BALB/c mice. Co-culturing these reporter cells with target cells infected with WT or uninfected cells of H-2^b^, H-2^a^, or H-2^kr^ origin, we observed high activation of Ly49C reporter cells (Fig. 3 C, left, and not depicted). An only slightly lower activation of Ly49C reporter cells was observed upon
co-culture with Δm04-infected BALB/c MEFs when compared with WT-infected targets (Fig. 3 C, left). However, co-culture of Ly49C reporter cells with MEFs of H-2k origin showed significantly lower activation of the Ly49C reporter cells co-cultured with Δm04-infected targets (Fig. 3 C, right). Thus, although the effect of m04 is most evident with Ly49A reporter cells, it is also visible with Ly49C reporter cells.

**m04 antagonizes the function of m152 in the downmodulation of H-2Dk**

To study the efficiency of the m04 gene product in the context of MCMV infection, we investigated the role of m04 alone, or in relation to two other MCMV MHC I regulators and used viral mutants individually lacking m04, m06, or m152, and different combinations of these three genes, or a triple mutant (Wagner et al., 2002). Contrary to the deletion of m04, which strongly reduced the activation of Ly49A reporter cells in the case of WT infection, the deletion of either m06 or m152 immunoevasins did not affect the capacity of infected cells to activate the reporter cell line (Fig. 4 A, bottom). As expected, the deletion of m06 and m152 together (Δm06Δm152), or the deletion of all three viral regulators of MHC I (Δm04Δm06Δm152) resulted in Ly49A reporter cell activation (Fig. 4 A, top). When the deletion of m04 was combined with a deletion of m06 (Δm04Δm06), the reporter cell activation was also lost, indicating that m152 alone is sufficient to down-regulate MHC I expression and prevent Ly49A engagement, when unopposed by m04. However, when both m04 and m152 were missing (Δm04Δm152), Ly49A reporter cells were activated to the same extent as the reporter cells co-cultured with noninfected cells. To elucidate this somewhat unexpected finding we followed the kinetics of surface H-2Dk down-regulation in cells infected with WT MCMV or mutants lacking m06, m04 alone, or in combination with the deletion of m06 or m152 genes (Fig. 4 B). We have observed differences in the kinetics of H-2Dk surface downmodulation, showing a more rapid and efficient activity of m152 (infection with Δm04Δm06) in comparison to m06 (infec tion with Δm04Δm152). In addition, these results confirm the previous findings of Holtappels et al. (2006), which showed that m04 antagonizes the MHC I retention function of m152 (as seen upon infection with Δm06). Only at 36 hpi does m06 induce levels of H-2Dk down-regulation comparable to those of m152. Therefore, given the results shown in Fig. 4 A, the absence of m04 in the case of Δm04Δm152 MCMV infection did not prevent Ly49A binding because of the differential kinetics of MHC I down-regulation by m06 and m152.

**Figure 3.** m04 allows engagement of inhibitory Ly49 receptor.

(A) Ly49A<sup>CD3</sup> or Ly49P<sup>CD3</sup> NFAT-GFP reporter cells were co-cultured with CBA/J, BALB/c, or C57BL/6 MEFs and either infected with indicated viruses or left uninfected. GFP expression was assessed (in the case of infected cells, overlay was made with uninfected cells presented by open histograms). Data are representative of two to four independent experiments. (B) Ly49A<sup>HCTS</sup> reporter cells were co-cultured with the indicated MEF cells, left uninfected, or infected with WT or Δm04 MCMV. GFP expression was assessed. Data are representative of two to four independent experiments. (C) Ly49C NFAT-GFP reporter cells were co-cultured with BALB/c MEFs and BALB/F MEFs that were infected with indicated viruses or left uninfected and GFP expression was assessed. P values, unpaired two-tailed Student’s t test. Data are means of four independent experiments. Error bars represent SD.
Ly49C\(^+\) and Ly49A\(^+\) NK cells results in a moderate control of \(\Delta m04\) MCMV, whereas the Ly49G2\(^+\) NK cell depletion results in a loss of control of the mutant virus. This suggests that Ly49G2\(^+\) NK cells play a dominant role in the control of \(\Delta m04\) MCMV replication.

The presence of \(m04\) impairs the proliferation of NK cells

To test whether the enhanced control of \(\Delta m04\) MCMV infection as opposed to WT MCMV infection is accompanied by increased proliferation and/or alterations in the size of NK cell sub-populations, we have infected BALB/c and BALB.K mice with WT and \(\Delta m04\) MCMV and monitored the proliferation of total NK cells by BrdU incorporation at 3 dpi. There was an enhanced incorporation of BrdU by NK cells upon infection with both viruses as opposed to uninfected controls. However, NK cell proliferation was impaired in both mouse strains after WT infection as compared with mice infected with \(\Delta m04\) MCMV (Fig. 5 B). Therefore, we have concluded that the presence of \(m04\) impairs the proliferation of NK cells, and this impairment is correlated with poor control of WT MCMV compared with the virus lacking the \(m04\) gene (\(\Delta m04\); Fig. 1).

Again, the impact of \(m04\) is more pronounced in BALB.K than in BALB/c mice, which corresponds to the more pronounced differences in viral titers between WT and \(\Delta m04\) observed in these mouse strains. No major differences in the proportion of various NK cell subpopulations expressing different inhibitory Ly49 receptors was observed.
between WT and Δm04 MCMV infection, suggesting that all three NK cell subsets contribute to overall increased NK cell proliferation after infection with Δm04 virus (Fig. 5 C). Indeed, this was visible after assessing BrdU incorporation by NK cell subpopulations expressing individual Ly49 inhibitory receptors (Fig. 5 D), both in BALB/c and BALB.K mice, although it was more pronounced in BALB.K mice.

The control of the virus lacking m04 is partially mediated via NKG2D

NK cells require an activating signal to respond to an insult, despite the absence of MHC I-mediated inhibitory signals. BALB/c mice do not possess an MCMV–specific, highly expressed, and strong activating receptor, such as Ly49H, which is present in C57BL/6 mice. Therefore, we tested whether NKG2D might play a role in the missing self-mediated recognition in mice infected with either WT MCMV or the Δm04 mutant (Fig. 6). In contrast to WT MCMV, blocking of NKG2D in H-2d or H-2k mice infected with Δm04 MCMV resulted in a significant increase in virus titer. This suggests that lowered inhibition leads to NK cell activation via stimulatory receptors capable of recognizing ligands expressed on infected cells.

We have not observed an effect of NKG2D blocking after infection with WT MCMV. This might be explained not only by NKG2D subversion mechanisms developed by MCMV, but also by a strong integration of inhibitory signals provided by m04–MHC I–mediated ligation of Ly49 receptors. Because m04 is not involved in the down-regulation of NKG2D ligands, one might wonder how NKG2D–blocking results in increased virus titer in Δm04 but not in WT MCMV–infected mice. We speculate that down-regulation of NKG2D ligands within different cell types in vivo is not as absolute as in permissively infected cells in vitro. To assess a possible contribution of another activating receptor, we performed a similar experiment in Not1−/− BALB/c mice (Gazit et al., 2006), but the results were comparable to the ones described in normal BALB/c mice. This indicated that NKP46 activating receptor does not participate in the missing self-mediated recognition of MCMV–infected cells (unpublished data).
In this study, we have described a mechanism by which MCMV evades NK cell–mediated control in spite of a marked down-regulation of MHC I proteins. By enabling MHC I molecules to escape complete down-regulation from the surface of infected cells, the m04 protein allows engagement of inhibitory Ly49 receptors, thereby preventing NK cell activation via the missing self process. Therefore, m04 might be a key player in determining the threshold level of NK cell inhibition. Our present findings are in accordance with recent studies demonstrating that the expression of inhibitory Ly49 receptors recognizing self–MHC I ligands restrict the proliferation and effector functions of Ly49H+ NK cells in response to MCMV infection in C57BL/6 mice (Orr et al., 2010).

The results obtained after NK cell subset depletion indicate the importance of a missing self recognition of virally infected cells by each of the NK cell subsets examined, although the stochastic expression of inhibitory Ly49 receptors precludes the exclusive depletion of one specific subset. We observed a loss of control of Δm04 MCMV replication in the absence of Ly49G2+ NK cells in BALB/c mice. The involvement of Ly49G2+ NK cells has been previously described to be important in the control of WT MCMV in C57L.M-H2b mice (Xie et al., 2009). The fact that Ly49G2+ NK cell–depleted mice infected with WT or Δm04 MCMV demonstrated increased viral titers could indicate that Ly49G2+ NK cells might be more easily triggered during infection and contribute to the control of viral spread. Indeed, previous work has demonstrated that Ly49G2+ NK cells preferentially express high-levels of IL12Rb2 subunit, which may render them more responsive to IL-12 during infection (Chakir et al., 2000). However, we have not observed significant differences in the percentage of IFN-γ+ NK cells expressing different inhibitory Ly49 receptors during WT MCMV infection (unpublished data). Furthermore, it has been shown that Ly49G2+ NK cells expand not only during MCMV infection but also during LCMV, mouse hepatitis virus, and vaccinia virus infection of C57BL/6 mice (Tay et al., 1999; Daniels et al., 2001). All these viruses modulate MHC I expression (Bruttikiewicz et al., 1992; Hewitt et al., 2002; Sevilla et al., 2004; Hahn et al., 2005; Dasgupta et al., 2007). Moreover, Ly49G2 lacks a ligand in mice bearing MHC I–deficient mice (Kavanagh et al., 2001b).

We observed that m04 is one of the most abundantly produced proteins of MCMV (unpublished data). Furthermore, a great number of polymorphic variants of m04 have been identified from MCMV strains isolated from wild mice, suggesting strong selective pressure (Smith et al., 2006). m04 in complex with H-2Dk molecules is essential for the recognition of infected cells by the activating Ly49P receptor (Kielczewska et al., 2009). m157, an MCMV glycoprotein recognized by the activating Ly49H and inhibitory Ly49I receptors, is also under intense selective pressure and easily mutated (Voigt et al., 2003; French et al., 2004). We propose that m04 originally evolved to function as an agonist of certain inhibitory Ly49 receptors and that the genesis of the activating Ly49P receptor recognizing m04 was a consequence of counteracting selective pressure in the host.

**DISCUSSION**

To avoid destruction by MHC I–restricted CD8+ T cells, many viruses have evolved mechanisms to modulate MHC I expression. However, infected cells down-regulating MHC I should be immediately recognized by NK cells because of the absence of inhibitory receptor engagement (e.g., Ly49s, KIRs, or CD94/NKG2A), thus shifting a balance of signals toward NK cell activation. MCMV encodes three regulators of MHC I expression. Although the function of m152 and m06 as negative regulators of MHC I expression is clear, the role of m04 remains enigmatic. Kleijnen et al. (1997) showed that m04 biochemically antagonizes the MHC I retention function of m152, a finding confirmed functionally in subsequent studies (Wagner et al., 2002; Holtappels et al., 2006), thus showing m04 as a positive regulator of MHC I cell surface expression and antigenic peptide presentation. However, it has also been proposed that the binding of m04 to MHC I proteins might interfere with the peptide loading onto MHC I or with the recognition by the TCR (Kavanagh et al., 2001b).

We observed that m04 is one of the most abundantly produced proteins of MCMV (unpublished data). Furthermore, a great number of polymorphic variants of m04 have been identified from MCMV strains isolated from wild mice, suggesting strong selective pressure (Smith et al., 2006). m04 in complex with H-2Dk molecules is essential for the recognition of infected cells by the activating Ly49P receptor (Kielczewska et al., 2009). m157, an MCMV glycoprotein recognized by the activating Ly49H and inhibitory Ly49I receptors, is also under intense selective pressure and easily mutated (Voigt et al., 2003; French et al., 2004). We propose that m04 originally evolved to function as an agonist of certain inhibitory Ly49 receptors and that the genesis of the activating Ly49P receptor recognizing m04 was a consequence of counteracting selective pressure in the host.
Mice used in experiments were housed in cages in a climate-controlled animal facility. Inbred strains of mice were maintained at the University of British Columbia (Vancouver, Canada), the University of California, San Diego (La Jolla, CA), and Washington University School of Medicine, St. Louis, MO. The construction of the Ly49D backbone was performed as described in the paper by Kielczewska et al. (2007). In brief, the C-terminal CTLD domain of the inhibitory Ly49 receptors was amplified by PCR and fused via an Esp3I (MBI Fermentas) restriction site (2007). For the reporter cell assay, a preferential coexpression of Ly49D and Ly49H activation was obtained. The chimeric receptor obtained with this approach acquires the ability to transmit activating signals upon ligation via the adapter protein DAP12. All constructs were verified by DNA sequencing.

The chimeric Ly49 receptors were transfected into 293FT cells using a standard procedure (Brune et al., 1999). For the reporter cell assay, a constant level of transfection was used. A subcloning strategy was employed to generate the chimeric proteins of inhibitory Ly49 CTLD with an activating Ly49H backbone. The ligation site resided in the transmembrane region. The intracellular domain and Ly49A CTLD-Stalk domain with Esp3I designed restriction sites at 5′ and 3′, respectively, subsequent Esp3I digestion and ligation (MBI Fermentas) such that the ligation site resided in the transmembrane region. The construction of the chimeric protein of inhibitory Ly49 CTLD with an activating Ly49H backbone was performed as described in the paper by Kidzewska et al. (2007). In brief, the C-terminal CTLD domain of the inhibitory Ly49 receptors was amplified by PCR, and fused via an Esp3I (MBI Fermentas) restriction site to the stalk, transmembrane region, and intracellular domain of the activating Ly49H. The chimeric receptor obtained with this approach acquires the ability to transmit activating signals upon ligation via the adapter protein DAP12. All cDNAs encoding the chimeric Ly49 receptors were subcloned into BamHI and NotI sites in the retroviral expression pMx-puro vector.

**MATERIALS AND METHODS**

**Mice and infection conditions.** Mice used in experiments were housed and bred under SPF conditions. All the protocols used for the breeding of mice and different kinds of treatment were approved by the Ethical Committee of the University of Rijeka and were performed in accordance with Croatian Law for the Protection of Laboratory Animals that has been harmonized with the existing EU legislation (EC Directive 86/609 EEC). Infections were performed by intravenous injection of the virus and, where indicated, NK cells were depleted by intraperitoneal injection of 20 µl of rabbit anti-asialo GM1 (Wako Chemicals) 24 h before infection. NKGD2 blockade was performed by injection of 300 µg of anti-NKGD2 antibody (C7 clone; Ho et al., 2002). Ly49 subsets were depleted by injections of 150 µg of YEE1/48 (anti-Ly49A), SE6 (anti-Ly49C and Ly49I), or 4D11 (anti-Ly49G2) antibodies (SE6 and 4D11 mAbs were a gift from W. Held, Ludwig Institute for Cancer Research Ltd., Lausanne Branch, Ealing, Switzerland; Sentman et al., 1989; Takei, 1983; Mason et al., 1995). The viral titers in organs were quantified by the standard plaque assay.

**Construction of chimeric Ly49 receptors.** The full-length cDNA of Ly49a was cloned into the pGEM-Teasy vector (Promega). Ly49a was a gift from F. Takei (University of British Columbia, Vancouver, Canada) and Ly49c was a gift from J.R. Carlyle (University of Toronto, Ontario, Canada). CD3ζ-NKR-P1A construct was a gift from W.M. Yokoyama (Washington University School of Medicine, St. Louis, MO). The construction of Ly49a-CD3ζ chimera was performed by PCR amplification of the CD3ζ intracellular domain and Ly49A CTLD-Stalk domain with Esp3I designed restriction sites at 5′ and 3′, respectively, subsequent Esp3I digestion and ligation (MBI Fermentas) such that the ligation site resided in the transmembrane region. The generation of the chimeric protein of inhibitory Ly49 CTLD with an activating Ly49H backbone was performed as described in the paper by Kidzewska et al. (2007). In brief, the C-terminal CTLD domain of the inhibitory Ly49 receptors was amplified by PCR, and fused via an Esp3I (MBI Fermentas) restriction site to the stalk, transmembrane region, and intracellular domain of the activating Ly49H. The chimeric receptor obtained with this approach acquires the ability to transmit activating signals upon ligation via the adapter protein DAP12. All cDNAs encoding the chimeric Ly49 receptors were subcloned into BamHI and NotI sites in the retroviral expression pMx-puro vector.

**Reporter cell assay.** B6F1 NTAT-GFP reporter cells were generated by retroviral transduction, as previously described (Arase et al., 2002). Primary CBA/J, BALB/c, BALB.K, BALB.F, and C57BL/6 MEFs were prepared by using a standard procedure (Brune et al., 1999). For the reporter cell assay, stimulator cells were infected (1.5 PFU/cell) with MCMV or MCMV deletion mutants (Wagner et al., 2002) for 12 h before the addition of reporter cells. NFAT-GFP reporter cells were co-cultured for 24 h with stimulators in 24-well plates at a ratio of 1:3 and analyzed by flow cytometry.

**Figure Legends**

**Figure 1.** Ly49a−/−NK cells contribute moderately to the control of the virus. In mice expressing H-2k, Ly49A lacks a ligand. In mice of the H-2k or H-2d background, Ly49A recognizes H-2Dk or H-2Dd, respectively (Karlihofer et al., 1992). Our recent studies using Ly49A reporter cells suggest more efficient recognition of H-2k rather than H-2d, a difference that is augmented by the presence of m04. As such, the absence of m04 should have greatest impact in mice expressing H-2k, intermediate impact in mice expressing H-2d, and no effect in mice bearing H-2b, as supported by both our in vivo and in vitro results. Furthermore, it has been shown that Ly49A+ NK cells are unable to reject H-2b bone marrow grafts, but they act synergistically with Ly49G2+ cells (Razziuddin et al., 2000).

Ly49C+ cells, on the other hand, represent almost 50% of the NK cell population in BALB/c mice, but contribute only moderately to the control of the virus. Ly49C is able to interact with H-2Kd and H-2Db in the H-2d background and, with H-2Kd and H-2Dd in the H-2d background, but only with H-2Dd in the H-2b background (Brennan et al., 1996b). Therefore, in the absence of m04 and reduced MHC I expression, NK cells of H-2k mice should show the greatest reduction in the inhibitory threshold. It is well established that MCMV encodes many MHC I–like molecules that are expressed on the surface of infected cells, and one can speculate that ligation of Ly49C by these molecules possibly results in the inhibition of Ly49C+ NK cell subset.

Ly49G2+ NK cells might be more efficient in detecting missing self, but inhibitory signals mediated by these cells might be weaker, as the strength of their interaction with MHC I is inferior to Ly49A, Ly49C, or Ly49I (Hanke et al., 1999). The robust response of Ly49G2+ NK cells raises the possibility of the existence of an activating receptor that is preferentially coexpressed on these cells and drives their proliferation. Because a preferential coexpression of Ly49D and Ly49H activation receptors has been shown (Smith et al., 2000), we cannot exclude the possible coexpression of an MCMV–specific activating receptor on these NK cells. We showed that NKGD2 receptor blocking resulted in a significant increase in the Δm04 virus titer, although it did not completely eliminate NK cell–mediated resistance by itself, suggesting the involvement of other activating NK cell receptors.

It has been shown that Ly49A− NK cell subsets are only weakly licensed in C57BL/6 mice (Brodin et al., 2009; Jonsson et al., 2010), which in the absence of an H-2b ligand is also expected for Ly49G2+ NK cells. Therefore, these cells are probably not operational in the missing self–mediated recognition of virally infected cells and this might be the reason why we have not observed the phenotype of Δm04 mutant in C57BL/6 mice. The quantitative differences of the m04 effect between H-2k and H-2d mice are not easy to explain in the absence of detailed knowledge of the binding of MHc I molecules for their cognate receptors. However, several nonexclusive mechanisms could be at play, including quantitative differences in the strength of licensing. A recent study by Jonsson et al. (2010) showing better licensing of Ly49A+ NK cells in mice of the H-2d haplotype supports this hypothesis. And yet, in our experiments shown in Fig. 3 (A and B), we observed that the impact of the absence of m04 was less dramatic in cells of H-2d background, both for Ly49A and Ly49C reporter cells, which could indicate the presence of residual Ly49 inhibition in H2d mice. Also, the fact that Ly49G2 possesses two H-2d ligands, H-2D and H-2L, in BALB/c mice but only one, H-2D, in BALB.K or C3H/J, would further contribute to an increased threshold of activation in H-2d mice infected with Δm04 MCMV.

Together, our findings highlight the importance of viral modulation of MHc I to prevent missing self signals from being detected by NK cells. This study presents the first evidence that NK cell recognition of the missing self is operational in the recognition and control of a viral pathogen in vivo.
MCMV and NKG2D expression analysis. Target B12 (H-2D^d) and SVEC4-10 (H-2K^d) cells were infected with 3 and 1.5 PFU/cell, respectively, of WT or Δm04 MCMV or were uninfected. Cells were harvested 10 hpi and stained for MHC I expression using anti-H-2D^d (clone 34-5–8S), anti-H-2K^d (clone SF1–11), anti-H-2L^d (clone 28–14–8), or anti-H-2D^P (clone 15–5–8S), followed by FITC-conjugated goat anti-mouse IgG (BD). Isotype-matched irrelevant antibodies were used as control. NKG2D ligand expression was analyzed using PE-labeled NKG2D tetramers (Krmptoci et al., 2002). PE-conjugated streptavidin (Bioscience) was used as a control staining antibody.

MHC I kinetics. MCMV-infected (1.5 PFU/cell) CBA/J MEFs were harvested at the indicated time points and stained with anti-H-2D^d (clone 15–5–8S), followed by FITC-conjugated goat anti-mouse IgG (BD).

Cytotoxicity assay. Target B12 (H-2^d) and SVEC4-10 (H-2^d) cells were infected with 3 and 1.5 PFU/cell, respectively, of WT or Δm04 MCMV, followed by centrifugal enrichment of infection. Uninfected cells were used as control. After 10-h infection, cells were labeled with CFSE and co-cultured with BALB/c or C3H splenocytes, respectively, at an effector-to-target ratio adjusted to the number of NK cells, where ratios 16:1, 8:1, and 4:1 roughly correspond to ratios 500:1, 250:1, and 125:1 of bulk splenocytes. After a 4-h co-culture in a 5% CO₂ atmosphere at 37°C, specific lysis was determined by flow cytometric analysis as measured by 7-aminoactinomycin D (7-AAD) incorporation (BD) in individual samples, as well as samples containing target cells only, to measure spontaneous death of cells. Cytotoxic activity was expressed as a specific lysis calculated by the following formula: (% CFSE^-7-AAD^+ cell specific lysis % CFSE^-7-AAD^+ cell spontaneous lysis / (100 - % CFSE^-7-AAD^+ cell spontaneous lysis) × 100. The experiment was done in triplicate for each effector-to-target ratio, and data points on the graphs represent the means.

Cell proliferation assay and immunofluorescence. BALB/c and BALB.K mice were injected with 2 × 10^5 PFU of WT or Δm04 MCMV and two and a half dpi received 2 mg of bromodeoxyuridine (BrdU). After 2 h BrdU stimulation isolated mouse splenocytes were either surface stained or BrdU incorporation isolated mouse splenocytes were either surface stained or BrdU incorporation stained for cell cycling, and BrdU incorporation isolated mouse splenocytes were either surface stained or BrdU incorporation isolated mouse splenocytes were either surface stained or BrdU incorporation isolated mouse splenocytes were either surface stained or BrdU incorporation.

Statistical analysis. Statistical significance was determined by GraphPad Prism5 software. Differences in viral titer between experimental groups were determined by the unpaired two-tailed Mann-Whitney test. Statistical significance of differences in the percentage of specific lysis and in BrdU proliferation assay was determined by the unpaired two-tailed Student’s t test.

Online supplemental material. Fig. S1 shows that the presence of m04 during WT MCMV infection protects RAW264.7 macrophage cell line from specific lysis. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20100921/DC1.

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