Distinct MHC class I–dependent NK cell–activating receptors control cytomegalovirus infection in different mouse strains

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Recognition of mouse cytomegalovirus (MCMV)–infected cells by activating NK cell receptors was first described in the context of Ly49H, which confers resistance to C57BL/6 mice. We investigated the ability of other activating Ly49 receptors to recognize MCMV-infected cells in mice from various H-2 backgrounds. We observed that Ly49P1 from NOD/Ltj mice, Ly49L from BALB mice, and Ly49D2 from PWK/Pas mice respond to MCMV-infected cells in the context of H-2d and the viral protein m04/gp34. Recognition was also seen in the H-2b and/or H-2f contexts, depending on the Ly49 receptor examined, but never in H-2b. Furthermore, BALB.K (H-2k) mice showed reduced viral loads compared with their H-2d or H-2b congenic partners, a reduction which was dependent on interferon γ secretion by Ly49L+ NK cells early after infection. Adoptive transfer of Ly49L+, but not Ly49L−, NK cells significantly increased resistance against MCMV infection in neonate BALB.K mice. These results suggest that multiple activating Ly49 receptors participate in H-2–dependent recognition of MCMV infection, providing a common mechanism of NK cell–mediated resistance against viral infection.

NK cells are the effector lymphocytes of the innate immune system (Vivier et al., 2008). They can recognize and spontaneously kill transformed or infected cells, a function which is regulated by the integration of a multitude of signals from both inhibitory and activating germline-encoded NK cell receptors (NKRs). NK cells have several cellular recognition mechanisms to distinguish between self and non-self (Lanier, 2005, 2008b). For one, they preferentially eliminate target cells that do not express normal levels of self-molecules, such as the highly polymorphic MHC class I proteins (H-2 class I in mice). Under normal circumstances, these molecules interact with inhibitory NKR, including the killer cell immunoglobulin-like receptors (KIRs) in humans and Ly49 receptors in rodents. Consequently, the target cell is recognized as self and no killing occurs, as ligand binding promotes the recruitment of phosphatases via an immunoreceptor tyrosine–based inhibition motif in the cytoplasmic tails of the receptors, generating an inhibitory signal. When surface expression of these self–molecules is disrupted, as is the case in some viral infections, the inhibitory signal is abrogated and the target cell is lysed. This method is known as “missing self” recognition (Ljunggren and Kärre, 1985). In parallel, the infection induces the synthesis of stress and viral proteins that are expressed at the cell surface, proteins which closely resemble self–molecules or are associated with them. Some activating NKRs are able to bind these stress molecules, whereas others recognize viral proteins as non-self. This recognition mechanism can be called “stressed self” or “altered self,” respectively. Activating NKRs lack intrinsic signaling activity and instead associate with adaptor proteins containing immunoreceptor tyrosine–based activation motifs, such as DAP10 or DAP12, which initiate signal transduction cascades leading to NK cell granule

Abbreviations used: IQR, interquartile range; KIR, killer cell immunoglobulin-like receptor; LAK cell, lymphokine-activated killer cell; MCMV, mouse cytomegalovirus (CMV); MEF, mouse embryonic fibroblast; NKR, natural killer domain; NKR, NK cell receptor; p.i., post infection; TM, transmembrane.
mobilization, cytokine secretion, and ultimately the killing of the target cell (Lanier et al., 1998; Orr et al., 2009; Tassi et al., 2009).

The critical role of activating NKR s in viral infections has been best characterized in acute infection with mouse CMV (MCMV). As other members of the β- Herpesviridae family, MCMV has evolved immune-evasion strategies that allow unrestricted viral replication in most inbred mouse strains during the early stage of infection (Scalzo et al., 1995). A few strains, however, are naturally resistant to MCMV infection. In particular, C57BL/6 (B6) mice express the Ly49H-activating receptor that binds to m157, a viral MHC class I homologue expressed at the infected cell surface during the early phase of infection. This event triggers NK cell activation and elimination of the infected cells (Arase et al., 2002; Smith et al., 2002). There is overwhelming evidence supporting the central role of the Ly49H–m157 axis in MCMV resistance. Indeed, Ly49H transgenesis into genetically susceptible mouse strains renders them resistant to MCMV. Conversely, knocking out the Ly49h or Dap12 genes in normally resistant animals abrogates this resistance (Sjölin et al., 2002; Cheng et al., 2008; Fodil-Cornu et al., 2008). In addition, B6 mice become susceptible to MCMV infection when challenged with a mutant MCMV virus lacking the m157 gene (Bubić et al., 2004).

Notably, a second NK cell–dependent mechanism of resistance to MCMV was found in MA/My mice. Indeed, the epistasis between the Ly49 and H-2 loci underlies this resistance (Desrosiers et al., 2005). In this model, the activating Ly49P receptor requires both host H-2Dk molecule and viral m04/gp34 protein to mediate recognition of MCMV-infected cells (Kielczewska et al., 2009). These results indicate that in addition to Ly49H, the Ly49P receptor mediates resistance to MCMV infection yet through a different process. Therefore, it has prompted us to explore whether other Ly49-activating receptors share the ability to recognize MCMV infection in either a Ly49H or Ly49P manner.

In mice, a vast repertoire of Ly49 receptors has been described. To date, four Ly49 haplotypes have been completely elucidated by genomic sequence analysis (Carlyle et al., 2008). Out of 15 Ly49 genes, B6 mice possess two that...
encode activating receptors (Ly49d and h). In contrast, the BALB/c strain has one activating receptor (Ly49i) out of seven Ly49 genes. In 129 mice, three activating receptors (Ly49r, u, and p) can be found among 12 Ly49 genes. Conversely, 7 out of 21 Ly49 genes are activating in NOD/Ltj mice (Ly49d, u, p, r, w, m, and h; Carlyle et al., 2008). As opposed to their inhibitory counterparts, rare self-ligands have been described for activating Ly49 receptors (Ly49P, Ly49R, Ly49W, Ly49D, Ly49U, and Ly49H) (et al., 2000, 2001). Although some are known to recognize viral proteins, many remain orphans.

In this paper, we demonstrate that the Ly49L-BALB-, Ly49P1-NOD-, and Ly49D2-PWK-activating receptors can recognize MCMV-infected cells in an m04/gp34-specific H-2-dependent manner. Among them is a novel activating receptor isolated from the wild-derived mouse strain PWK/Pas. Moreover, the improved ability of BALB.K (H-2b) mice to control MCMV proliferation in the spleen relative to BALB/c (H-2b) or BALB.By (H-2k) mice is NK cell dependent. In addition, we show that it correlates with the specific expansion and IFN-γ secretion from Ly49L+ NK cells in these mice. Finally, the survival of neonate mice after MCMV infection is increased upon adoptive transfer of Ly49L+ NK cells. These results suggest that the m04/gp34-specific H-2-dependent detection of infected cells by activating Ly49 receptors is a common mechanism of host defense, whereas Ly49H-m157 recognition remains restricted to B6 mice.

RESULTS

Multiple activating Ly49 receptors recognize an MCMV-infected cell based on the presence of the m04/gp34 viral peptide and of a specific H-2 context

Given the close relationship between MCMV and its host, we examined the ability of activating Ly49 receptors to respond to MCMV-infected cells in different H-2 contexts. For this, we cloned 13 activating Ly49 receptors into 2B4 cells expressing the M2-tagged DAP12 adaptor protein. Equivalent Ly49 expression and functionality in reporter cells was assessed with α-M2 antibody (unpublished data). Reporter cells were co-cultured with a panel of mouse embryonic fibroblast (MEF) cells of different H-2 haplotype (H-2b, H-2k, H-2d, H-2r, H-2b, H-2r, H-2b, H-2k, H-2b, H-2b, and H-2b) under various conditions (Fig. 1 and Table I). As expected, Ly49H reporter cells were stimulated by MCMV-infected MEFS independently of the H-2 background as a result of the presence of the viral molecule m157 on the surface of infected cells (Arase et al., 2002). No stimulation was observed for Ly49D-BALB-, Ly49D-NOD-, Ly49M-NOD-, Ly49RMA/My-, Ly49UMA/My-, and Ly49D1-PWK-bearing 2B4 cells under any of the conditions tested (Table I). Ly49W1 reporter cells were stimulated MEF cells of H-2b, H-2k, or H-2b haplotype irrespective of the condition tested (Fig. 1A). In contrast, in addition to Ly49PMA/My, three other reporter cell lines, Ly49PBALB (Ly49L), Ly49PNOD (Ly49P1), and Ly49D2-PWK (Ly49D2), were stimulated both in an MCMV- and H-2-dependent fashion. However, the extent of functional recognition for each receptor was different. Ly49P1-expressing cells were weakly stimulated by uninfected or infected H-2d MEFs but responded robustly by MCMV-infected cells of the H-2b background. Ly49D2 reporter cells were only stimulated by infected H-2d MEFs. Ly49L reporter cell activation was MCMV dependent in multiple H-2 contexts, with the strongest activation observed in H-2b (~60%), intermediate in H-2k (~50%), and weak in H-2d (< 40%) contexts (Fig. 1A).

To examine the role of the m04/gp34 molecule in receptor recognition, reporter cells were co-cultured with MEFS infected with two different deletion MCMVs. The first deletion lacked the ORF encoding m04/gp34 alone (Δm04), whereas the second lacked three ORFs encoding m04/gp34, m06/gp48, and m152/gp40 (Δm04Δm06Δm152; Fig. 1A). The m06/gp48 and m152/gp40 products are immunoevasins that down-regulate MHC class I expression (Jorići et al., 2008). Therefore, cells infected with mutant Δm04Δm06Δm152...
MCMV express high levels of MHC class I molecules as opposed to WT or Δm04-infected cells (Fig. S1). Ly49H reporter cells were stimulated by MEFs infected with both WT and MCMV mutant viruses. Ly49W1 reporter cells were stimulated in all conditions, further demonstrating that their activation depends on the presence of H-2 molecules rather than MCMV viral proteins. In contrast, the absence of m04/gp34 effectively reduced the activation of Ly49P1, Ly49D2, or Ly49L reporter cells even with high levels of MHC class I expression (Fig. 1 A). To examine the involvement of the MHC class I molecules, reporter cell assays were carried out in the presence of anti-H-2Dk, anti-H-2Kk, or control IgG antibodies (Fig. 1 B). Stimulation of the reporter cells was abolished specifically in the presence of anti-H-2Dk antibodies, demonstrating that the ability of Ly49D2, Ly49P1, and Ly49L to recognize MCMV-infected cells depends on the presence of the H-2Dk molecule. Thus, we identified three additional haplotype-specific Ly49 stimulatory receptors that share a common H-2–dependent recognition mechanism of MCMV-infected cells with Ly49PMA/My2B4 reporter cells involving the m04/gp34 viral protein and H-2Dk molecules.

Structurally, activating receptors that recognize MCMV-infected cells conditional on their H-2 background belong to group II (because of the absence of predicted helix α3 within loop L3; Deng et al., 2008). The amino acid sequence alignment of their natural killer domain (NKD) regions revealed three common features shared by four receptors that were identified beforehand. Those include a threonine to arginine/methionine substitution at position 224 resulting in the loss of a glycosylation motif at position 221–223, as well as a conserved leucine and asparagine residues at position 234 and 244, respectively (Fig. 1 C). Whether and how these residues influence MCMV recognition remain to be established.

NK cell–mediated control of MCMV replication in BALB mice depends on H-2 haplotype

BALB mice possess the smallest described Ly49 repertoire, with only four Ly49 receptors expressed on mature NK cells (Ly49A, C, G, and L; Ortaldo et al., 1999; Van Beneden et al., 2001; Gayès et al., 2006). Moreover, the availability of BALB animals congenic for different H-2 loci offers the opportunity to examine in vivo the role of Ly49L+ NK cells in H-2d, H-2b, or H-2k contexts. At a dose of 5 × 10^3 PFU, viral replication rapidly progressed in BALB.K (H-2k) mice, reaching Log_{10} 5 ± 0.1 PFU at 2 d post infection (p.i.) However, starting at day 4, viral load decreased, culminating at Log_{10} 3 ± 0.2 PFU by day 10 p.i. This reduction was not seen at the same level in BALB/c (H-2d) mice, which showed viral titers 50-fold higher than those of BALB.K mice by day 6 p.i. and were moribund by day 10 p.i. (Fig. 2 A). At the same dose, BALB.By (H-2b) mice succumbed between days 3 and 4 p.i. (not depicted); however, even upon infection with half the normal dose (2.5 × 10^3 PFU), they had a significantly higher viral load than BALB.K mice by day 4 p.i. (Fig. 2, A and B). Interestingly, the MCMV viral load in the liver of BALB.K mice was fourfold lower by day 4 p.i. than in BALB.By mice (Fig. 2 B), yet the viral load difference between BALB.K and BALB/c mice only became significant...
increased virus titers although with different patterns. The effect of NK cell depletion was seen from day 4 to 10 p.i. in the spleen, but it became apparent in the liver at later time points (days 6 and 10 p.i.; Fig. 3, A and B, top). In contrast, the effect of CD8$^+$ T cell depletion was observed earlier in the liver (days 4 and 6) and later in the spleen (days 6 and 10) over the course of infection (Fig. 3, A and B, bottom). Interestingly, splenic viral titers continued to decrease over the course of the infection after either NK cell or CD8$^+$ T cell depletion, demonstrating that both lymphocyte populations contribute to viral clearance from day 6 p.i. onward. Indeed, when both cell populations were depleted at the same time, a >100-fold increase in viral titers was observed in all organs tested by day 6 p.i. and mice were moribund shortly afterward (Fig. 3 C). CD4$^+$ T cells have also been shown to play a role during the chronic phase of MCMV infection, especially in the salivary gland (Polić et al., 1998; Lee et al., 2009; Andrews et al., 2010). We thus depleted BALB.K mice of CD4$^+$ T cells and infected them with WT or Δm04 MCMV. Their spleens were collected at different time points p.i. (C and D). The number of NK cells (C) and the percentages of Ly49A-, Ly49C-, Ly49G-, or Ly49L-positive NK cell fractions (D) were determined. Asterisks denote p-values <0.05. (E) Representative dot plots of Ly49A and Ly49L expression on NK cells from BALB.By, BALB/c, or BALB.K mice infected with WT MCMV at day 4 and 6 p.i. Data are from three experiments (mean ± SD) with three to four mice per group. n/a, not available.
Table II. Contribution of Ly49L+ NK cells to the production of IFN-γ 36 h and 6 d p.i. after MCMV infection

| Mouse strain and condition tested | Percentage of total IFN-γ+ NK cells | Percentage of Ly49L+ NK cells |
|----------------------------------|-------------------------------------|------------------------------|
| BALB/By Uninfected               | 1.52 ± 0.40                         | 12.33 ± 3.07                 |
| +MCMV (36 h p.i.)                | 14.55 ± 2.13                        | 17.33 ± 6.76                 |
| +MCMV (Day 6 p.i.)               | 2.26 ± 0.24                         | 16.50 ± 1.27                 |
| BALB/c Uninfected                | 1.99 ± 0.873                        | 10.69 ± 1.44                 |
| +MCMV (36 h p.i.)                | 13.60 ± 3.70                        | 13.37 ± 1.18                 |
| +MCMV (day 6 p.i.)               | 5.4 ± 1.28                          | 23.47 ± 9.00                 |
| BALB/K Uninfected                | 1.63 ± 0.79                         | 10.13 ± 2.58                 |
| +MCMV (36 h p.i.)                | 13.45 ± 4.67                        | 11.65 ± 5.26                 |
| +MCMV (day 6 p.i.)               | 9.06 ± 2.07                         | 53.6 ± 2.0                   |

In this table, we show the percentage of IFN-γ-producing NK cells among the total CD3+DX5+ splenocyte pool at two different time points after infection (percentage of total). The contribution of the Ly49L+ NK cells to the production of IFN-γ was calculated by dividing the percentage of Ly49L+IFN-γ+ by the total percentage of IFN-γ+ NK cells. Data represent the percent mean ± SD of three mice per group from one of two experiments performed.
in the BALB model, we examined NK cell production of IFN-γ at 36 h and 6 d p.i. with MCMV. At the initial time point, intracellular staining of IFN-γ after restimulation revealed a similar frequency of IFN-γ–producing cells (~20%) in the BALB. By, BALB/c, and BALB.K strains. Within this subset of cells, the Ly49L+ NK fraction represented only a minority of total NK cells, that is to say ~15% (Fig. 5 A and Table II). By day 6 p.i., IFN-γ production by NK cells decreased significantly in all strains, although this decrease was the least pronounced in BALB.K mice (Fig. 5). This correlates with our observation that Ly49L+ NK cells were the main IFN-γ producers in BALB.K mice. Conversely, the Ly49L+ NK cells from BALB/c and BALB.By mice were responsible for only a small fraction of IFN-γ production in these strains, although this proportion was significantly higher in BALB/c (Fig. 5, B and C; and Table II). Therefore, whereas there is nonspecific IFN-γ production by NK cells at 36 h p.i., preferential IFN-γ secretion by Ly49L+ NK is observed by day 6 p.i. after restimulation in BALB.K mice.

**Naive Ly49L+ NK cells protect neonate BALB.K mice against MCMV infection**

The transfer of adult naive Ly49H+ NK cells into neonatal mice is sufficient to protect against MCMV infection (Bukowski et al., 1985; Sun et al., 2009). Thus, to investigate the protective potential of Ly49L+ NK cells we used adoptive transfer into 3–5-d-old pups 1 d before MCMV infection (Fig. 6 A). We negatively selected NK cells, sorted Ly49L+ (YE1/48 INT 12A8−) or Ly49L− (YE1/48 12A8−) NK cells from adult BALB.K female mice, and injected various numbers of each cell fraction in pups (Fig. 6 B). Neonates injected with PBS alone were used as negative control, whereas transfer of NK cells from resistant MA/My mice were used as positive control. Neonates receiving the Ly49L− NK cell fraction were not protected and, similar to the PBS-treated group, succumbed by day 8 p.i. regardless of the cell number injected (Fig. 6 C and not depicted). In contrast, 64% of neonatal mice receiving 10⁵ NK cells from MA/My survived the infection. Protection conferred by Ly49L+ NK cells was dose dependent. Survival of infected mice receiving 4 × 10⁴ Ly49L+ NK cell fraction was increased by ~1 wk compared with 10⁴ NK cell transfer, even though none of the neonates survived. Markedly, 40% of mice receiving 7 × 10⁴ Ly49L+ NK cells survived 3 wk p.i. (Fig. 6 C). Finally, at the same time, the transfer of Ly49L+ NK cell fraction significantly reduced viral titers in the spleens of neonates compared with Ly49L− NK cell transfer or PBS-injected controls (Fig. 6 D). Thus, the Ly49L+ NK cell fraction restricted the viral load and was ultimately more protective than Ly49L− NK cells against MCMV infection.

**DISCUSSION**

A protective role of Ly49-activating receptor during virus infection has been amply demonstrated in B6 mice, whose NK cells are activated through an Ly49H–m157 axis (Lanier, 2008a). Another NK cell–dependent mechanism of MCMV resistance, which has been associated with the ability of activating Ly49P receptor to recognize H-2Dk and m04/gp34 on the MCMV-infected cell, has been described in MA/My mice (Desrosiers et al., 2005; Kielczewska et al., 2009). Although the involvement of Ly49P was validated in vitro, the same cannot be said for in vivo studies because no appropriate antibody was available to properly delimit the Ly49P population.
Furthermore, those two Ly49-activating receptors recognize infected cells by different mechanisms, raising the question of whether a finite number of Ly49-activating receptors is used to tackle many different pathogens or whether there is a preferential specificity of certain activating receptors for MCMV. Our results revealed that the latter seems to be the case. Reporter cells expressing Ly49P1NOQ, Ly49LBALB, or Ly49D2PWK recognize MCMV-infected MEFs. Remarkably, their mode of recognition is H-2-dependent, with a preference for H-2ª and sometimes for H-2ª and H-2ª haplotypes, requiring the presence of the viral m04/gp34 protein. Thus, these three Ly49 haplotype-specific receptors are capable of recognizing MCMV infection in an H-2Dª- and m04/gp34-restricted manner, along with Ly49P.

Amino acid sequence alignment of the activating Ly49 receptors assayed in our study has yielded new clues into the mechanism of m04/gp34- and H-2-dependent MCMV recognition. Three residues were found to be conserved among activating Ly49 receptors capable of recognizing the infection and absent in those that are not: methionine/arginine (M/R) at position 223, and leucine (L) and asparagine (N) residues at position 234 and 244, respectively. Interestingly, the two latter are localized to β3 and β4 sheets, which are predicted to be involved in the binding with the H-2 class I molecules. Moreover, M/R at position 223 removes an N-glycosylation (NTT) site shared by the receptors that do not recognize infection. Previous studies have shown that glycosylation at this site affects Ly49 receptor binding to H-2 class I molecules. For example, a mutation in Ly49A promoting glycosylation at NTT (221–223) prevented H-2D8-tetramer binding, whereas alteration of this motif in Ly49D and the subsequent loss of glycosylation increased its ability to bind the tetramer (Mason et al., 2003). The importance of receptor glycosylation has also been observed in T lymphocytes. Indeed, mice deficient for the enzyme that performs N-glycosylation have enhanced TCR clustering and a lower activation threshold. During CD8+ T cell development, glycosylation of the CD8 β-chain decreases avidity to H-2 class I molecules (Demetriou et al., 2001; Moody et al., 2001). Thus, it is possible that lack of a functional glycosylation motif facilitates binding of the activating Ly49 receptors to H-2 class I molecules on MCMV-infected cells. Of course, the specific involvement of the M/R, L, and N residues in the recognition of MCMV-infected cells will have to be assessed through site-directed mutagenesis and direct binding assays of soluble forms of the receptors. Nevertheless, the available data suggest that these residues are in direct contact with the H-2 class I molecule rather than with viral m04/gp34 protein, which remains essential for this mechanism of recognition.

The m04/gp34 protein is thought to stabilize the H-2 on the surface of infected cells; however, m04/gp34 has not yet been shown to directly interact with activating Ly49 receptors on the surface of infected cells. Furthermore, we have previously shown that m04/gp34 is necessary but not sufficient for recognition of infection by Ly49PIMA/NM-expressing reporter cells, yet it remains to be established whether an additional host or pathogen factor is also involved in viral recognition by the three receptors described in this paper (Kielczewska et al., 2009). Hence, this viral protein might induce an allosteric change or cluster H-2 molecules on the surface of infected cells, thus allowing a longer and stronger interaction with activating Ly49 receptors. The questions remain of which is the additional host or viral molecule in addition to m04/gp34 that is necessary to promote recognition of the infected cell by activating Ly49 receptors, and whether it is shared by all the receptors. Then again, is it a stress or viral peptide produced during infection, which is part of the H-2D8–gp34 complex or its presentation by specific H-2 molecules? Most studies show that Ly49 receptors recognized H-2 molecules independently of the peptide being presented under normal conditions. Yet some receptors might be sensitive to a possible peptide-induced allosteric change in H-2 (Correa and Raulet, 1995; Orihuela et al., 1996; Hanke et al., 1999).

The prevalence of the m04/gp34-dependent method of viral recognition suggests an important role for this viral peptide in immunoevasion. We have recently shown that the Δm04 virus is attenuated in most susceptible strains, including BALB.K, as the protein it encodes abolishes NK cell activation via the missing-self recognition mechanism (Babić et al., 2010). Indeed, m04/gp34 escorts MHC class I molecules to the surface of infected cells, thus maintaining a level of surface MHC expression sufficient enough to trigger several inhibitory NKRs (Kavanagh et al., 2001; Babić et al., 2010). For instance, Ly49A-bearing reporters recognize uninfected cells; however, a much stronger, m04-dependent response is observed upon co-culture of these reporters with MCMV-infected cells.

Therefore, in BALB.K mice both activating and inhibitory Ly49 receptors are triggered by m04/gp34–H-2 complexes. However, as the frequency of Ly49L+ NK cells is low, initially the predominant signal is m04-dependent inhibition. In the case of m04/gp34 ablation, NK cell inhibition is removed allowing enhanced early control of virus replication even if Ly49L+ NK cell activation is also absent.

Our next step was to investigate the role of these three activating receptors in vivo. Experiments focusing on the Ly49L receptor were particularly telling. For instance, 15% of NK cells in uninfected BALB mice express the activating Ly49L receptor. Yet after MCMV infection, the kinetic analysis of the Ly49 repertoire demonstrated a specific expansion of the Ly49L+ NK cell fraction starting at day 4 p.i. in BALB.K mice, which was not the case in BALB. By mice; in this case, BALB/c mice displayed an intermediate phenotype. Interestingly, no such Ly49L+ NK cell proliferation was observed upon infection with Δm04 MCMV, indicating that Ly49L+ NK cells need to sense the m04/gp34 product to respond to infection. The expansion was correlated with an increased ability to control the infection, as well as increased IFN-γ secretion by H-2ª Ly49+ NK cells after day 6 p.i. This was also seen in H-2ª mice, although to a much lesser extent, and was entirely absent in H-2ª mice. All in all, these findings demonstrate that Ly49L+ NK cells in the context of H-2ª are specifically activated during MCMV infection. In another
experiment, adoptive transfer of a Ly49L+ cell fraction significantly extended the life span of neonate pups after MCMV infection, a protection not seen when a Ly49L− fraction was transferred. In this assay, naive Ly49L+ NK cells provide in vivo control of viral load. Recently, rechallenged NK cells have been shown to proliferate better and to secrete more IFN-γ than their naive counterparts (Cooper et al., 2009; Sun et al., 2009). In an adoptive transfer experiment, low numbers of rechallenged NK cells provided protection from MCMV, whereas naive NK cells did not. A dose-dependent protection by the naive Ly49L+ NK cells can also be seen; indeed, the transfer of 7 × 10^4 of those cells was able to improve mice survival to the same extent as a transfer of 10^5 total naive NK cells from resistant MA/My mice. Therefore, the transfer of Ly49L+ NK cells isolated from mice previously infected with MCMV (i.e., memory NK cells) could provide better protection to neonate mice than the transfer of naive NK cells. Collectively these results point to a central role of the activating Ly49L receptor in the control of viral spread. By analogy with the response of Ly49H+ NK cells during infection, one possible interpretation of these results is that Ly49L+ NK cells are being activated through a Ly49L–m04/gp34–H-2Dk axis, leading to elimination of MCMV-infected cells.

Despite having an activating receptor capable of recognizing the infection, viral titers in the spleen of BALB.K mice are elevated during the initial phase of the infection. This could be a result of three important facts. First, the Ly49L+ NK cell population is small in the absence of infection, only accounting for ~15% of total splenic NK cells. Therefore, the initial inability of BALB.K to control MCMV proliferation could simply be the result of an insufficient number of effector cells. In comparison, 28% of splenic NK cells express Ly49H in Tg915 mice (transgenic for Ly49H). This is enough to make them significantly less resistant to MCMV infection than B6 mice, in which 50% of total splenic NK cells are Ly49H+ (Lee et al., 2003). Second, surface expression of m04/gp34 is dependent on its association with H-2, yet H-2 is rapidly modulated by other viral immune evasion signals such as m152/gp40 and m06/gp48, which, respectively, retain it in the cis-Golgi compartment of the endoplasmic reticulum or redirect it to endosome–lysosome pathway for degradation (Kleijnen et al., 1997; Ziegler et al., 1997). This can limit the amount of available ligand with which Ly49L can interact. In contrast, m157, the ligand for Ly49H, is ubiquitously expressed starting at 4 h p.i. Third, competition with other inhibitory Ly49 receptors expressed on the same NK cell (e.g., Ly49C and Ly49G) could alleviate activation mediated by Ly49L+ NK cells (Babić et al., 2010). These inhibitory signals might overcome Ly49L signaling and drive the NK cell to unresponsiveness rather than activation. At later time points, though, the specific expansion of Ly49L+ NK cells may amplify the activating signal to a point where it overwhelms inhibition and induces NK cell activation, which also serves to initiate the adaptive immune response.

Ly49G and Ly49A are known to bind to H-2Dk and H-2Dd molecules (Chung et al., 2000; Silver et al., 2002). Thus, Ly49A+ and/or Ly49G+ NK cells will only receive inhibitory signals through these receptors in those two H-2 contexts. Therefore, expansion of these NK cell subpopulations—in the H-2k context specifically—could result in nonspecific NK cell activation. This may be mediated by an inflammatory milieu that is not balanced by the inhibitory signals of Ly49A and Ly49G in the absence of their ligands. Furthermore, the Ly49G+ NK cell fraction has been shown to expand in C57BL/6 mice upon MCMV, vaccinia virus, mouse hepatitis virus, and lymphocytic choriomeningitis virus infection, as well as in MA/My mice upon MCMV infection (Daniels et al., 2001; Xie et al., 2009; Babić et al., 2010). Moreover the increase in this NK fraction could be a negative regulatory mechanism to control activation of NK cells.

Even though the protection against MCMV infection conferred by Ly49L was restricted to the H-2Dk context, our in vitro analysis showed that Ly49L–expressing 2B4 reporter cells were able to recognize MCMV infection in the H-2d context. BALB/c mice were more resistant than BALB.By mice, as shown by the equivalent splenic viral titers at any given time point, even though the BALB/c mice were challenged with twice the viral dose. We also detected an expansion of the splenic Ly49L+ NK cell subpopulation, as well as greater secretion of IFN-γ upon restimulation at day 6 p.i. in the BALB/c strain. Although it was not the purpose of our study, one might speculate that the inhibitory NK cell pathways differ among H-2 congenic BALB strains. Specifically, the threshold of NK cell activation might be higher in BALB.By and BALB/c mice than in BALB.K mice as a result of more potent and overwhelming pathways of NK cell inhibition. Whether this is actually the case remains to be determined.

In addition to Ly49L, other activating receptors were considered in this study, including Ly49D2, which was isolated from the resistant wild-derived strain PWK/Pas. Haplotype analysis showed significant similarity between PWK/Pas and B6 at Ly49h locus, yet NK cells in PWK/Pas animals were negative for Ly49H. Moreover, PWK/Pas mice are completely resistant to infection with ∆m157 MCMV, as opposed to B6 mice (Adam et al., 2006). However, MCMV resistance in PWK/Pas is determined by a major locus, Cmv4, with only a small contribution of H-2. Furthermore, the endogenous H-2^paw haplotype is the susceptibility allele, and Ly49D2 reporter cells are not stimulated in the endogenous H-2^paw background. All of this suggests the involvement of other receptors in the resistance phenotype. Regardless of the identity of Cmv4, our results indicate the presence of at least one Ly49-activating receptor in each of the five Ly49 haplotypes capable of recognition of MCMV infection. It is noteworthy that in four examples the mechanism of recognition implicates a host class I molecule, indicating that such is a common mechanism of Ly49-activating receptor recognition as opposed to direct binding to a viral protein as afforded by Ly49H.

Although MCMV recognition is at the core of this paper, some activating receptors were found to recognize uninfected MEF cells. We identified a new endogenous ligand for the Ly49W<sup>NOD</sup> receptor, H-2<sup>d</sup>, in addition to the previously
identified H-2D\(\alpha\) and H-2D\(\delta\) molecules (Kane et al., 2001). Thus, although Ly49W-expressing reporter cells were not stimulated by H-2\(\delta\) MEFs, the endogenous NOD H-2 haplotype, autoimmunity or anergy could result from having an activating NKR with affinity for a cognate self-H-2 molecule. Recently, it has been shown that anergy is the most likely outcome. For instance, chimeric mice constitutively expressing m157 display hyporesponsive Ly49H\(^{+}\) NK cells after MCMV infection (Sun and Lanier, 2008; Tripathy et al., 2008). However, the physiological relevance and the functional role of the activating Ly49W receptor, capable of binding to self H-2 molecules, need to be further studied, namely in NOD mice congenic for H-2\(^{a}\) or H-2\(^{b}\). Nevertheless, it seems that the impact of Ly49W on NK cell homeostasis would not be affected in NOD mice.

The present study has improved our grasp of NK cell-mediated recognition of MCMV in a mouse model. Undoubtedly, parallels can be made with HCMV infection in humans. However, KIRs, which have a homologous function to Ly49 receptors, have not been directly implicated in HCMV-infected cell recognition. Yet NK cell involvement is undeniable. For instance, individuals with NK cell deficiencies are hypersusceptible to infection with herpesviruses, HCMV among them (Biron et al., 1989; Orange et al., 2002). Furthermore, HCMV seropositive patients have increased circulating levels of NKG2C\(^{+}\) NK cells compared with uninfected individuals; upon in vitro co-culture with HCMV-infected fibroblasts, the NKG2C\(^{+}\) NK cell fraction specifically proliferates (Gumá et al., 2004, 2006). Moreover, a significantly reduced risk of HCMV reactivation was reported in sibling transplantations where the donor had more than one activating KIR (Cook et al., 2006). Individuals with different KIR repertoires respond differentially to infection with other viruses such as HIV. For example, the activating KIR3DS1 receptor mediates host protection to HIV infection when its ligand HLA-Bw4-80I is expressed on infected cells (Martin et al., 2002). KIR3DS1\(^{+}\) NK cells were also shown to specifically expand during acute HIV-1 infection given the presence of HLA-B Bw4-80I (Alter et al., 2009).

Ancient viruses, namely CMVs, have coevolved with their host–pathogen interaction led to the emergence of numerous combinations of both activating and inhibitory NKRs and MHC class I molecules to control infection. We propose that human NK cell–mediated resistance to MCMV | Pyzik et al.

### MATERIALS AND METHODS

**Mice.** BALB.By, BALB.K, BALB/c, B10.M-H2\(\text{f}\)/nMobl, B10.RIII-H2\(\text{e}\)/71NSnMobl, FVB/NJ, C57BL/6, and A/J mice were purchased from The Jackson Laboratory. NOD/LtJ and PWK/Pas mice were provided by C.A. Piccirillo (McGill University, Montreal, Canada) and F. Colucci (University of Cambridge, Cambridge, UK), respectively. Animal protocols or experiments were approved by the Canadian Council on Animal Care and the McGill University Animal Resources Center. Unless otherwise specified, all animals were between 8 and 10 wk of age. Donor mice were 8–12 wk old.

**Cloning.** Ly49H, Ly49C, Ly49P, Ly49U, and Ly49R, reporter cells were generated as previously described (Kielczewska et al., 2009), Ly49A, Ly49D, and Ly49G from C57BL/6, Ly49A, Ly49L, and Ly49G from BALB, Ly49R from NOD/LtJ, and Ly49D1 and Ly49D2 from PWK/Pas were cloned into pMX-puromycin vectors using specific primers (Table S1) as previously described (Arase et al., 2002). 2B4 reporter cells were generated as previously described (Fodil-Cornu et al., 2010). Chimera protein receptors were generated by joining the NKG2D from each aforementioned inhibitory Ly49 to the backbone (cytoplasmic/transmembrane [TM] stalk: CTS) of activating Ly49H as described by Kielczewska et al. (2007). The CD3\(\zeta\)-Ly49A\(^{TM}\)-NK-R1A construct was a gift from W.H. Yokoyama (Washington University School of Medicine, St. Louis, MO). Using this, a Ly49A-CD3\(\zeta\) chimera was generated in a threefold manner. First, the CD3\(\zeta\) and the Ly49A NKD-stalk domain were amplified by PCR from their respective vectors. Specific primers that also allowed the partial amplification of the Ly49A TM domain in each construct (located in the 5’ end and in the 3’ end for the CD3\(\zeta\) and Ly49A constructs, respectively) and the addition of an Esp3I restriction site were used. Second, both PCR products were digested with Esp3I and ligated (Thermo Fisher Scientific) together as follows: CD3\(\zeta\)-Ly49A\(^{TM}\)-Esp3I and Esp3I-Ly49A\(^{TM}\)/NOD/NKG (ligation site in the Ly49A TM domain).

**Virus stocks, mouse infections, and virus titration.** The Smith strain of MCMV was obtained from the American Type Culture Collection. Salivary gland stocks were prepared by propagation in 3-wk-old BALB/c mice as previously described (Desrosiers et al., 2005). 7–8-wk-old mice were injected i.p. with 5 \(\times\) 10\(^5\) or 2.5 \(\times\) 10\(^5\) PFU/mouse of MCMV or tissue culture grown 5 \(\times\) 10\(^5\) PFU/mouse of MCMV. Viral titers in various organs were assessed by plaque assay as previously described (Desrosiers et al., 2005). For NK and/or CD8 T cell depletion experiments, mice were injected i.v. with either 30 \(\mu\)l of α-asialo-GM1 (Wako Chemicals USA) or 250 \(\mu\)g of α-CD8 mAb (clone: HJ5-17.2.4; gift from M. Pauvre, Centre d’Immunologie de Marseille-Luminy, Marseille, France) 48 h before infection. Viral titers were assessed at days 4 and 6 p.i. To assess viral titers at day 10, mice were injected one additional time at day 4 p.i. As plaque assays were performed, depletion was assessed by FACs. The MCMV strain used was MCMV 152, and WT BAC-derived (MW97.01) viruses were previously described (Wagner et al., 1999).

**Flow cytometry, NK cell enrichment, and sorting.** Antibodies used were the following: NKp46 (29A1.4; FITC, PE, or Alexa Fluor 647 conjugated; ebioScience), Pan-NK cells (DX5; FITC, PE, or Alexa Fluor 647 conjugated; ebioScience), Ly49A (YE1-48; FITC, PE, or biotin conjugated and purified; BioLegend), Ly49A/D (12A8; PE conjugated; ebioScience), Ly49G (4D11; FITC or PerCP-eFluor710 conjugated; ebioScience), Ly49G (AT8; PE conjugated; ebioScience), Ly49C (14B11; FITC or biotin conjugated; ebioScience), Flag-M2 (Sigma-Aldrich), donkey α-mouse IgG (polyclonal; PE conjugated; ebioScience), IFN-γ (XMG1.2; PerCP-Cy5.5 conjugated; ebioScience), CD3ε (145-2C11; PerCP-Cy5.5 conjugated; ebioScience), CD8a (53–6–7; FITC or PE conjugated; ebioScience), TCR-β (HSY-9-797; FITC conjugated; ebioScience), H-2D\(^\delta\) (15–5–8; unconjugated or FITC conjugated; ebioScience), H-2D\(^\delta\) (34–2–12; FITC conjugated; ebioScience), H-2K\(^\text{b}\) (SF1-1.1; FITC conjugated; ebioScience), H-2K\(^\text{b}\) (36–7–15; unconjugated or FITC conjugated; ebioScience), IgG2a isotype control (biotin conjugated; ebioScience), and streptavidin (FITC, PE, APC, PerCP-Cy5.5, or eFluor450 conjugated; ebioScience). Splenocytes from infected mice were collected in 4 ml of 2% DME and macerated with cell strainers. 3/4 of the sample was used to assess the viral titer via plaque assay, and 1/4 was treated with Ack’s lysis buffer, washed in cold PBS, and stained for various specific surface markers. In particular, the best demarcation of the Ly49L/NKD cell fraction was seen after surface staining and a mild 15-min fixation in 4% paraformaldehyde (Stewart et al., 2007).
To enrich NK cells and/or to FACS sort them, spleens were harvested from BALB mice, perfused with cold PBS and macerated. The resulting cell suspension was treated with ACK5 lysing buffer and resuspended in PBS with 10% FBS. Next, the sample was negatively enriched in NK cells with the NK cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. In the case of adaptive transfers, the negative fraction (>75% NK cell purity) was stained with specific surface markers and sorted with the FACSArria (BD). For IFN-γ staining, 2 × 10^5 enriched NK cells or splenocytes/well were plated in 96-well plates and activated with 5 μg/ml of plate-bound NKp46 (R&D Systems) and 1 μg/ml NKGD2 (clone: A10; eBioscience) mAb for 4 h. Alternatively, cells were stimulated with 50 μg/ml PMA/1 μg/ml ionomycin (Sigma-Aldrich). Intracellular markers were stained according to the manufacturer's conditions (Cytofix/ CytoPerm Plus Fixation/Permeabilization kit with GolgPlugi BD). Cells were acquired on a FACScalibur (BD), FAScCanto II (BD), or CyAn (Beckman Coulter) and analyzed by FlowJo (7.6;Tree Star) analysis program.

Adaptive transfers. 3–5-d-old BALB.K suckling mice were used as recipients. Before use in experiments, neonates from different litters were pooled together and randomly reassigned, with a maximum of nine pups per lactation female. Groups of four to nine mice were given an ip injection with 50 μl of given cell population. The next day, mice were infected with 10^3 PFU per pup of salivary gland MCMV in a volume of 50 μl. Mice were monitored daily for survival. Some were killed 3 or 6 d later, and their spleens and livers removed and used for virus titration or FACS analysis as described in the previous sections. Controls were neonates injected with PBS or total MACS enriched NK cells from resistant MA/My mice.

Cell culture. MEFs were prepared as previously described (Desrosiers et al., 2005). Reporter cell assay and H-2 blocking studies were performed as previously described (Desrosiers et al., 2005). LAK cells were prepared as previously described (Podili-Cornu et al., 2008).

Statistics. Differences between groups were calculated with one-way ANOVA analysis assuming not-repeated measures, followed by Bonferroni post tests. Otherwise, a two-tailed unpaired Student's t test was used. Results with P < 0.05 were considered significant.

Online supplemental material. Fig. S1 shows MHC class I expression during MCMV infection. Fig. S2 shows hepatic MCMV proliferation in BALB mice. Fig. S3 shows that depletion of CD4+ T cells has no influence on MCMV viral titers in BALB.K mice in spleen or liver. Fig. S4 shows Ly49 repertoire in BALB congenic mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20101831/DC1.

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