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The Role of LY49 NK Cell Subsets in the Regulation of Murine Cytomegalovirus Infections

Chin H. Tay, Lawrence Y. Y. Yu, Vinay Kumar, Llewelyn Mason, John R. Ortaldo, and Raymond M. Welsh

The distributions and functions of NK cell subsets, as defined by the expression of Ly49 NK cell receptors, were examined in murine CMV (MCMV)-infected mice. MCMV induced a reduction in NK1.1+ cell number in the spleen and an increase in the peritoneal exudate cells. Within the splenic NK1.1+ population, proportional increases in Ly49A+ and Ly49G2+ cells but decreases in Ly49C+ and Ly49D+ cells were observed 3 days post-MCMV infection, but within the peritoneal NK1.1+ cell populations there were proportional decreases in Ly49A+ cells and increases in Ly49C+, Ly49D+, and Ly49G2+ cells. Lymphocytic choriomeningitis virus did not elicit a comparable NK cell subset distribution. Lymphokine-activated killer cells were sorted into different Ly49 NK cell subsets and adoptively transferred into C57BL/6 suckling mice. Regulation of MCMV synthesis in these suckling mice was shown to be an IFN-γ-dependent, perforin- and Cmv-1-independent process, and each NK cell subset mediated anti-viral activity. In adult C57BL/6 mice, the control of MCMV in the spleen is mediated by a perforin-dependent mechanism, regulated in part by the Cmv-1 gene, which maps closely to the Ly49 family. In vivo deletions of either one or two of the Ly49 subsets in adult mice did not affect the ability of the residual NK cells to regulate MCMV synthesis. These data provide evidence of NK cell subset distribution and function in MCMV infection, but no individual subset was required for the Cmv-1-like regulation of MCMV synthesis. The Journal of Immunology, 1999, 162: 718–726.
To address this possibility, we examined 1) the distribution of Ly49 NK cell receptors in the spleens and peritoneal cavities of uninfected and 3-day MCMV-infected C57BL/6 mice, 2) the mechanisms of the control of MCMV in C57BL/6 suckling mice adoptively reconstituted with NK cells, 3) the activity of NK cells of different Ly49 subsets to protect the suckling mice, and 4) the regulation of MCMV infections in adult C57BL/6 mice treated with mAbs to the different members of the Ly49 family.

Materials and Methods

Cells

YAC-1, a highly NK-sensitive lymphoma line, was cultivated in suspension in RPMI 1640 (Sigma, St. Louis, MO), supplemented with 10% FBS, l-glutamine, and antibiotics. Mouse embryonic fibroblasts (MEF) from C57BL/6 mice and vero cells (monkey kidney cells) were cultivated as monolayers in MEM (Life Technologies, Grand Island, NY) with l-glutamine, antibiotics, and 20 or 100% FBS, respectively.

Mice

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). These mice were conventionally housed, bred in our animal facility, and used at 6–12 wk of age. C57BL/6 suckling mice were used at 4–6 days of age in the adoptive transfer experiments. Breeder pairs of 129 wild-type control and 129 mice homozygous for a targeted mutation disrupting the mouse IFN-γ gene (IFN-γR<sup>−/−</sup>) were obtained from Dr. M. Aguet (University of Zurich, Switzerland) (25). Breeder pairs of 129 × C57BL/6 mice heterozygous for a targeted mutation disrupting the gene for perforin were originally derived and kindly provided by Drs. C. M. Walsh and W. R. Clark (University of California, Los Angeles, CA) (26). F<sub>1</sub> offspring of the above heterozygous breeding pairs were used. NH<sub>4</sub>SO<sub>4</sub>-cut anti-Ly49D ascites, 12A8, was produced in BALB/c mice. MCMV was titrated by plaque assay on MEF cells and Ly49 NK cell subsets and decreases in the percentages of Ly49C<sup>+</sup> cells was evaluated by the ANOVA test.

Immunoﬂuorescence

Phycoerythrin-labeled anti-NK1.1 mAb, FITC-labeled anti-Ly49A mAb, and FITC-labeled Ly49C mAb were purchased from PharMingen (San Diego, CA). These Abs were used according to manufacturer's speciﬁcations. FITC-labeled anti-Ly49D (12A8) and FITC-labeled anti-Ly49G2 (4D11) were used at 50 µl of 1:80/10<sup>6</sup> cells and 50 µl of 1:40/10<sup>6</sup> cells, respectively. The anti-Ly49D (12A8) Ab used in the in vivo depletion studies and the adoptive transfer studies was cross-reactive with the Ly49A NK cell receptor (20). However, a different FITC-labeled anti-Ly49D Ab (4E5), which was not cross-reactive with Ly49A receptor, was used in the study of NK cell distribution. This Ab was used at a concentration of 50 µl of a 1:1000 dilution/10<sup>6</sup> cells. The significance of differences in proportions of Ly49 subpopulations within NK1.1<sup>+</sup> cells was evaluated by the ANOVA test.

Results

Distribution of NK1.1<sup>+</sup> Ly49<sup>+</sup> NK cell subsets in the spleens of uninfected and 3-day MCMV-infected C57BL/6 mice

To examine if there were any differences in the distribution of the NK cell subsets before and after MCMV infection, spleen cells from uninfected and 3-day MCMV-infected C57BL/6 mice were stained for NK1.1 and for Ly49A, C, D, and G2 NK cell receptors. The virus dose used in all the experiments was shown previously to be sufficient to activate the NK cells (12). Compared with uninfected mice, MCMV infection did not alter the number of spleen leukocytes (data not shown). However, FACS analyses based on 10<sup>6</sup> gated lymphocytes showed that there were fewer NK1.1<sup>+</sup> cells (two- to threefold decrease) in the spleens of MCMV-infected mice compared with that of uninfected mice (Fig. 1 and Table I).

Adoptive transfer experiments were done by using a modification of a method previously described (6). Briefly, 4- to 6-day suckling mice were pooled and randomly assigned to lactating females. Groups of four to nine mice were given 5 × 10<sup>6</sup> LAK cells/mouse or 5 × 10<sup>3</sup> mouse spleen cells/mouse i.p. in 0.1 ml of media using a 1-ml syringe and a 30-gauge needle. The following day, the suckling mice were challenged with MCMV i.p. Three days postinfection, the mice were sacrificed, and the spleens were removed for virus titration.

Impaired NK cell function in infected mice

The virus dose used in all the experiments was shown previously to be sufficient to activate the NK cells (12). Compared with uninfected mice, MCMV infection did not alter the number of spleen leukocytes (data not shown). However, FACS analyses based on 10<sup>6</sup> gated lymphocytes showed that there were fewer NK1.1<sup>+</sup> cells (two- to threefold decrease) in the spleens of MCMV-infected mice compared with that of uninfected mice (Fig. 1 and Table I).

Analyses of the Ly49 NK cell subset distribution within the splenic NK cell population per 10<sup>6</sup> lymphocytes before and after MCMV infection revealed that there were increases in the percentages of Ly49A<sup>+</sup> (Expt. 1, 15–17%; Expt. 2, 19–25%; Expt. 3, 17–51%; Expt. 4, 20–27%; p = 0.2) and Ly49G2<sup>+</sup> (Expt. 1, 39–54%; Expt. 2, 43–55%; Expt. 3, 57–70%; Expt. 4, 44–58%; p = 0.001) NK cell subsets and decreases in the percentages of Ly49C<sup>+</sup> (Expt. 1, 45–23%; Expt. 2, 40–16%; Expt. 3, 48–30%; Expt. 4, 41–31%; p = 0.008) and Ly49D<sup>+</sup> (Expt. 1, 45–40%; Expt. 2, 42–29%; Expt. 3, 61–45%; Expt. 4, 45–39%; p = 0.03) NK cell subsets (Fig. 3). This pattern of the Ly49 subsets was not observed at 8 days post-MCMV infection (data not shown), and control mice injected with uninfected salivary gland extract also did not produce any of the changes seen in 3-day MCMV-infected mice (data not shown). These results suggest that, compared with uninfected mice, 3-day MCMV infection of C57BL/6 mice resulted in a decrease in the total number of NK1.1<sup>+</sup> cells and changes in the percentages of Ly49<sup>+</sup> NK cell subsets within the spleen.
Distribution of NK1.1<sup>+</sup>-Ly49<sup>+</sup> NK cell subsets in the peritoneal cavity of uninfected and 3-day MCMV-infected C57BL/6 mice

Analyses of the NK1.1<sup>+</sup>-Ly49<sup>+</sup> NK cell subsets in the spleen indicated that even though the absolute number of splenocytes was similar between uninfected and infected mice, there were decreases in the number of NK1.1<sup>+</sup> cells and changes in the proportion of the NK1.1<sup>+</sup>-Ly49<sup>+</sup> subsets after MCMV infection. To examine if there were any changes in the NK cell population in the peritoneal cavity, peritoneal exudate cells (PEC) from uninfected and 3-day MCMV-infected mice were stained with Abs to NK1.1, Ly49A, C, D, and G2 NK cell receptors. Compared with the uninfected controls, there was usually about a twofold increase in the absolute number of PEC in MCMV-infected mice (data not shown). FACS analyses showed that there were significantly more NK1.1<sup>+</sup> cells per 10<sup>4</sup> lymphocytes (two- to fourfold increase) in the peritoneal cavity after infection (Table I and Fig. 2). These data suggest that either there is an influx of NK1.1<sup>+</sup> cells into the peritoneal cavity during MCMV infection or else the resident NK cells are proliferating. FACS analyses of the Ly49 NK cell subsets within the NK1.1<sup>+</sup> population showed that there was a decrease in the percentage of Ly49A<sup>+</sup> cells (Expt. 1, 34–26%; Expt. 2, 36–32%; Expt. 3, 45–21%; Expt. 4, 37–23%; p = 0.06) cells while there were increases in the percentages of Ly49C<sup>+</sup> (Expt. 1, 34–46%; Expt. 2, 44–52%; Expt. 3, 30–46%; Expt. 4, 51–52%; p = 0.06), Ly49D<sup>+</sup> (Expt. 1, 24–50%; Expt. 2, 37–52%; Expt. 3, 26–53%; Expt. 4, 41–52%; p = 0.02), and Ly49G2<sup>+</sup> (Expt. 1, 46–61%; Expt. 2, 47–57%; Expt. 3, 46–60%; Expt. 4, 53–62%; p = 0.006) cells while there were decreases in the percentages of Ly49B<sup>+</sup> and Ly49D<sup>+</sup> cells, in the spleen. This was accompanied by the concomitant decrease in the percentage of Ly49A<sup>+</sup> PECs and increase in the percentage of Ly49C<sup>+</sup> and Ly49D<sup>+</sup> PECs. In both the spleen and the peritoneal cavity, the percentage of Ly49G2<sup>+</sup> cells increased after MCMV infection.

Distribution of NK1.1<sup>+</sup>-Ly49<sup>+</sup> NK cells in the spleen and peritoneal cavity in uninfected and 3-day LCMV-infected C57BL/6 mice

To determine whether the above mentioned pattern of NK cell accumulation only occurred during MCMV infection, C57BL/6 mice were infected with a different virus, LCMV, and spleen cells and PECs were then stained with the various NK cell receptor Abs. There were few differences in the absolute number of splenic leukocytes and PECs from 3-day LCMV-infected mice compared with uninfected mice (data not shown). FACS analyses also showed that there were few differences in the number of NK1.1<sup>+</sup> cells in the spleen (Table I), and in only one of three experiments there was an increase in NK1.1<sup>+</sup> cells per 10<sup>4</sup> lymphocytes in the peritoneal cavity (Table I). The distribution of the various Ly49 NK cell receptors in the spleen and the peritoneal cavity did not follow a reproducible pattern after 3 days of LCMV infection (Fig. 4). There were few to no changes in the percentages of Ly49A<sup>+</sup>, Ly49C<sup>+</sup>, and Ly49D<sup>+</sup> cells in the spleen, while the changes in the percentage of these three cells were erratic in the peritoneal cavity. However, there was an increase in the percentage of Ly49G2<sup>+</sup> NK cell subsets in both the spleen and the peritoneal cavity (spleen: Expt. 1, 39–62%; Expt. 2, 43–58%; Expt. 3, 36–58%; p = 0.02; and peritoneal cavity: Expt. 1, 46–60%; Expt. 2, 47–48%; Expt. 3, 45–58%; p = 0.2) (Fig. 4). Thus, the distribution of Ly49 NK cell subsets after MCMV infection is distinct for that particular NK-sensitive virus infection, thereby suggesting that the Ly49 NK cell subsets may play a role in the regulation of MCMV in C57BL/6 mice.

Effects of adoptively transferred Ly49<sup>+</sup> LAK cells on the regulation of MCMV in suckling mice

Three-week-old or younger suckling mice are very susceptible to MCMV infections, because NK cells take about 3 wk from birth to reach maturity (4, 5). Bukowski et al. have shown that the adoptive transfer of syngeneic adult spleen cells or LAK cells into suckling mice can protect them from a subsequent normally lethal MCMV infection (6). Analyses of splenic MCMV titers indicated that suckling mice adoptively reconstituted with adult spleen cells or LAK cells on the day of birth have lower MCMV titers compared with control suckling mice infected with the LCMV strain (data not shown), indicating that even though the absolute number of splenocytes was similar between uninfected and infected mice, there were decreases in the number of NK1.1<sup>+</sup> cells and changes in the proportion of the NK1.1<sup>+</sup>-Ly49<sup>+</sup> subsets after MCMV infection.
Table I.  Frequency of NK1.1c cells in the spleens and the peritoneal cavities of uninfected, MCMV-infected and LCMV-infected C57BL/6 mice

<table>
<thead>
<tr>
<th></th>
<th>Spleenb cells per 10⁴ lymphocytes</th>
<th>Peritoneal Cavityb cells per 10⁴ lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NK1.1c</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Expt. 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>285 ± 23</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>MCMV-infected</td>
<td>101 ± 8</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>LCMV-infected</td>
<td>294 ± 20</td>
<td>p = 0.6</td>
</tr>
<tr>
<td>Expt. 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>349 ± 38</td>
<td></td>
</tr>
<tr>
<td>MCMV-infected</td>
<td>108 ± 6</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>LCMV-infected</td>
<td>283 ± 9</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>Expt. 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>341 ± 51</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>MCMV-infected</td>
<td>149 ± 29</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Expt. 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>188 ± 22</td>
<td>p &lt; 0.5</td>
</tr>
<tr>
<td>LCMV-infected</td>
<td>155 ± 29</td>
<td>p = 0.6</td>
</tr>
</tbody>
</table>

a Age-matched C57BL/6 mice were either uninfected or given i.p. 1 × 10⁴ PFU/MCMV per mouse or 5 × 10⁴ PFU/LCMV per mouse.

b Spleen cells and peritoneal cavity cells were removed from uninfected, 3-day MCMV-infected, or 3-day LCMV-infected C57BL/6 mice and stained with anti-NK1.1 phycoerythrin mAb. A lymph gate was used to analyze the cells.

c The number of NK1.1c cells was based on 10⁴ lymphocytes.

d p value is calculated by comparing the number of NK1.1c cells from infected mice to the number of NK1.1c cells in the uninfected controls within the same experiment.

Splenocytes depleted of T cells do confer protection. Bukowski et al. also showed that much lower cell numbers of NK1.1+ LAK cell populations could protect suckling mice from MCMV and that neither adult splenocytes nor LAK cells protected suckling mice from the NK cell-resistant virus, LCMV (6). To examine the importance of the different Ly49 subsets in the early regulation of MCMV, we tested the ability of NK1.1+, Ly49+ LAK cells to protect suckling mice from an MCMV infection. LAK cells were generated using 6- to 12-wk-old C57BL/6 spleen cells. Fig. 5 shows the typical distribution of the different Ly49 subsets within the NK1.1+ LAK cell population. The percentages of the different Ly49 NK cell subsets in the LAK cell cultures were similar to the percentages seen in spleen NK cells taken from naive C57BL/6 mice as shown here and previously by others (18–20, 29). Before adoptive transfers, the LAK cells were sorted into NK1.1+–Ly49− or NK1.1+–Ly49− for all the four Ly49 subsets (A, C, D, and G2). These sorted LAK cells were adoptively transferred into 4- to 6-day-old C57BL/6 suckling mice, which were subsequently infected with MCMV, and splenic MCMV titters were measured 3 days later. Sucking mice adoptively reconstituted with any of the different combinations of NK1.1+ LAK cells had lower splenic MCMV titters compared with the media control (Table II). For example, both NK1.1+–Ly49A− LAK cells and NK1.1+–Ly49A− LAK cells significantly decreased the amount of MCMV in the spleen of the suckling mice compared with the media control (Table II, Expt. 2). It should be noted that a hundred times more naive adult splenocytes is required to see the same level of protection afforded by LAK cells (6). These results indicate that subpopulations of NK cells expressing any of the Ly49 NK cell receptors could protect the sucking mice from MCMV.

Effects of adoptively transferred C57BL/6 or 129 adult spleen cells and LAK cells on the regulation of MCMV in C57BL/6 suckling mice

The mechanisms by which adoptively transferred NK cells protect suckling mice from MCMV had not been previously determined. Experiments were performed to determine the mechanisms of MCMV control in the suckling mice and also to see if it was related to the Cmv-1 resistance gene. As shown previously, C57BL/6 leukocytes or C57BL/6 LAK cells when transferred into the suckling mice significantly lowered MCMV titers in the spleen compared with the media controls (Table III), and it required 5 × 10⁷ splenic leukocytes but only 3 × 10⁵ LAK cells to mediate protection (6). Interestingly, 129 spleen cells and LAK cells when adoptively transferred into C57BL/6 suckling mice also had a protective effect in the spleen compared with the media controls (Table III, Expts. 1 and 2). These results are surprising in view of the fact that the 129 strain is Cmv-1−, and the regulation of spleen MCMV titers in adult C57BL/6 mice is Cmv-1-dependent (12). Therefore, these data suggest that at 3 days post-MCMV infection, the protection afforded by the Ly49-bearing LAK cells in the spleens of C57BL/6 suckling mice is not Cmv-1-dependent.

Effects of adoptively transferred C57BL/6 or perforin 0/0 mice adult spleen cells and LAK cells on the regulation of MCMV in C57BL/6 suckling mice

Cmv-1-dependent, NK cell regulation of MCMV in the spleen of adult mice is mediated by a perforin-dependent mechanism (12). The results presented above suggested that the regulation of spleen titers of MCMV in the sucking mouse was not Cmv-1-dependent, but the role of perforin was not evaluated. Therefore, the abilities of perforin 0/0 spleen cells and perforin 0/0 LAK cells to control MCMV synthesis in the spleens of C57BL/6 suckling mice were tested. Perforin 0/0 spleen cells or LAK cells when adoptively transferred into 4- to 6-day-old C57BL/6 suckling mice significantly lowered MCMV titers in the spleens compared with control spleens (Table III, Expts. 3–5). The levels of protection afforded by perforin 0/0 spleen cells and LAK cells were similar to that of C57BL/6 spleen cells and C57BL/6 LAK cells, respectively. Therefore, results presented in Table IV suggest that adoptively transferred spleen cells or LAK cells protect C57BL/6 suckling mice from MCMV via a Cmv-1-independent, perforin-independent mechanism.
Effects of adoptively transferred C57BL/6 spleen cells on the regulation of MCMV in 129 and IFN-γ R0/0 mice

NK cells can regulate MCMV in adult mice livers via the production of IFN-γ and it was possible that the transferred NK cells controlled splenic MCMV titers in the suckling mice via the production of IFN-γ by the NK cells introduced into the peritoneal cavity. To test for this, adult C57BL/6 spleen cells were adoptively transferred into 4- to 6-day-old 129 or IFN-γ R0/0 suckling mice, and their abilities to reduce MCMV synthesis in the spleens were measured. In C57BL/6 suckling recipients, adult C57BL/6 spleen cells significantly reduced splenic MCMV titers compared with infected control spleens (Table IV, Expts. 1 and 2), but they had no effect on MCMV splenic titers in MCMV-infected IFN-γ R0/0 suckling mice. These data suggest that the control of MCMV synthesis in suckling mice by adoptively transferred cells is IFN-γ dependent. Thus, the numerous experiments listed in Tables III and IV collectively suggest that the control of spleen MCMV titers in suckling mice by adoptively transferred NK cells is Cmv-1- and perforin-independent but dependent on IFN-γ.

Effects of adoptively transferred C57BL/6 spleen cells on the regulation of MCMV in 129 and IFN-γ R0/0 mice

As the role of the innate resistance gene Cmv-1 has been best described in the adult mouse/MCMV model (12), adult C57BL/6 mice were used to test the hypothesis that the regulation of MCMV was dependent exclusively on any one of the Ly49 subsets defined by the four available Ly49 Abs. Individual Ly49 NK subsets or a combination of Ly49 subsets were depleted with mAbs in vivo, and NK cell activity and MCMV titers in the spleen and the liver were then measured in the adult mice 3 days postinfection. As mentioned in Materials and Methods, the anti-Ly49C and anti-Ly49G2 Ab stocks were titrated in vivo by measuring the rejection of parental bone marrow cells by Ly49C+ and Ly49G2+ cells in F1 (b × d) mice (18, 19). The anti-Ly49A and anti-Ly49D Abs were also previously titrated in vivo, and the depletions of the respective NK cell subsets were checked by FACS analysis. Depletion of any single Ly49 NK cell subset (Ly49A, Ly49C, Ly49D, or Ly49G2 alone) had very little effect on NK cell cytotoxicity, in contrast to the near complete NK cell depletion in MCMV-infected mice treated with anti-NK1.1 mAb (Fig. 6, A–C). This suggests that the residual NK cells can rapidly compensate for the depletion in any NK cell subset after virus infection. When compared with the infected controls, MCMV-infected C57BL/6 mice treated with either anti-Ly49A, C, D, or G2 mAbs alone exhibited no increase in virus titers in the spleen and the liver (Table V, Expts. 1–3). Depletion of two subsets of Ly49+ NK cells (Ly49C and Ly49D,
Ly49C and Ly49G2, Ly49D and Ly49G2) caused some reduction in NK cell cytotoxicity (Fig. 6, D and E), but these depletions had no effect on the regulation of MCMV in the spleen and the liver (Table V, Expts. 4 and 5). In two separate experiments, depletion of three Ly49+ NK cell subsets (Ly49C, Ly49D, and Ly49G2) resulted in diminished NK cell activity (Fig. 6), but in only one experiment did such a depletion result in an increase of MCMV titers in the spleen and in one other experiment it caused an increase in liver titers (Table V, Expts. 5 and 6). As a positive control in all the experiments, C57BL/6 mice treated with anti-NK1.1 mAb to deplete the NK cells exhibited the expected increase in MCMV titers in the spleen and the liver. These results indicated that in adult C57BL/6 mice the residual NK cells can compensate for the deletion of any one of the defined subsets for which Abs are currently available.

**Discussion**

This is the first demonstration that a virus infection can influence the distribution of NK cell subsets within and between different organs. There were fewer NK1.1+ cells in the spleen and more NK1.1+ cells in the peritoneal cavity after MCMV infection (Table I), but these changes in NK1.1+ cell numbers in the spleen and peritoneal cavity were not apparent during LCMV infection. As i.p. MCMV infection resulted in increases in both the absolute number of PEC and NK1.1+ cells in the peritoneal cavity, these

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**Table II. Control of MCMV by NK1.1+ LAK cells in MCMV-infected C57BL/6 suckling mice**

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Group</th>
<th>Log$_{10}$ PFU MCMV/Spleen</th>
<th>p$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Media control</td>
<td>3.3 ± 0.2</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Unsorted LAKs</td>
<td>4.0 ± 0.2</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>NK1.1+ Ly49A- LAKs</td>
<td>4.0 ± 0.2</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>3</td>
<td>Media control</td>
<td>4.1 ± 0.1</td>
<td>3.1 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>NK1.1+ Ly49C- LAKs</td>
<td>3.5 ± 0.5</td>
<td>3.5 ± 0.5</td>
</tr>
<tr>
<td>4</td>
<td>Media control</td>
<td>3.1 ± 0.0</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>NK1.1+ Ly49D- LAKs</td>
<td>2.4 ± 0.1</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>6</td>
<td>Media Control</td>
<td>3.1 ± 0.4</td>
<td>2.3 ± 0.3</td>
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<tr>
<td></td>
<td>NK1.1+ Ly49G2- LAKs</td>
<td>2.2 ± 0.5</td>
<td>2.2 ± 0.5</td>
</tr>
</tbody>
</table>

$^d$ Four- to six-day-old C57BL/6 suckling mice were given i.p. 2000 PFU MCMV per mouse.

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**Table III. Mechanisms of MCMV regulation in adoptively reconstituted C57BL/6 suckling mice**

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Group</th>
<th>No. of Cells Transferred</th>
<th>Log$_{10}$ PFU MCMV per Spleen ± SD$^c$</th>
<th>p$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Media control</td>
<td>129 spleen cells</td>
<td>5 x 10$^7$</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Media control</td>
<td>C57BL/6 spleen cells</td>
<td>5 x 10$^7$</td>
<td>3.3 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>Media control</td>
<td>129 LAK cells</td>
<td>5 x 10$^7$</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Media control</td>
<td>C57BL/6 LAK cells</td>
<td>5 x 10$^7$</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>3</td>
<td>Media control</td>
<td>Perforin 0/0 spleen cells</td>
<td>5 x 10$^7$</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Media control</td>
<td>C57BL/6 spleen cells</td>
<td>5 x 10$^7$</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>4</td>
<td>Media control</td>
<td>C57BL/6 spleen cells</td>
<td>5 x 10$^7$</td>
<td>3.4 ± 0.2</td>
</tr>
<tr>
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<td>Media control</td>
<td>Perforin 0/0 LAK cells</td>
<td>5 x 10$^7$</td>
<td>2.6 ± 0.4</td>
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<tr>
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<td>C57BL/6 LAK cells</td>
<td>5 x 10$^7$</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>5</td>
<td>Media control</td>
<td>Perforin 0/0 LAK cells</td>
<td>5 x 10$^7$</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Media control</td>
<td>C57BL/6 LAK cells</td>
<td>5 x 10$^7$</td>
<td>2.2 ± 0.2</td>
</tr>
</tbody>
</table>

$^c$ Four- to six-day-old C57BL/6 suckling mice were given i.p. 2000 PFU MCMV per mouse.

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$^c$ Age-matched C57BL/6 mice were given 0.1 ml complete RPMI 1640 per mouse (Media control) or were adoptively reconstituted i.p. with spleen cells or LAK cells in 0.1 ml complete RPMI 1640 at the cell concentration shown in the table. All these transfers were performed 1 day prior to infection.

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$^d$ Splenic MCMV PFU were titrated on C57BL/6 MEF 3 days after infection.

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$^p$ values were generated by comparing the virus titers from adoptively reconstituted groups to the media control.
results indicate that there either is proliferation of NK cells at the sites of infection or that there is trafficking of NK cells to those sites.

There are not only changes in NK cell numbers but also changes in the Ly49 subsets within the NK population. Even though MCMV did not cause any apparent change in the absolute number of splenocytes, the virus infection caused an increase in the percentage of Ly49A and Ly49G2 NK cell subsets but a decrease in the percentage of Ly49C and Ly49D NK cell subsets. Interestingly, these changes in the proportion of NK cell subsets within the spleen are accompanied by decreases in the percentages of the Ly49A NK cell subset and increases in the percentages of Ly49C, D, and G2 NK cell subsets in the peritoneal cavity. As this change of Ly49 subsets only occurs during MCMV infection but not during LCMV infection, the different Ly49 NK cell subsets may have different roles in the regulation of MCMV in vivo. The differences in the Ly49 distribution after MCMV or LCMV infection may be due to the ability of the virus infection to stimulate different cytokine profiles. MCMV but not LCMV has been shown to stimulate the production of IL-12 and IFN-γ 3 days postinfection (30, 31), and these cytokines may stimulate the production of chemokines that would attract the NK cells to the sites of infection. Another possibility is that the different Ly49 NK cell subsets may recognize MCMV itself or MCMV peptide/s-modified MHC molecules, such that the NK cells specifically home to the sites of infection.

Sucking mice less than 2 wk of age can be used as a model to study the role of NK cells in the regulation of MCMV because NK cells take about 3 wk from birth to reach maturity. In this model, spleen cells or culture-purified LAK cells when transferred into the sucking mice protect the mice from a lethal MCMV infection (6). These adoptive transfer studies cannot be performed in adult mice, as these mice would have to be irradiated to destroy the recipient’s immune system before the transfer. This is because resident NK cells in the adult mice have been shown to eliminate adoptively transferred LAK cells, preventing such adoptive transfer experiments to be performed in adult mice (32).

The regulation of MCMV in the spleen of adult mice is Cmv-1- and perforin_dependent, but in sucking mice, results indicate that this innate resistance gene and perforin do not play a role in the regulation of MCMV by adoptively transferred NK cells. In sucking mice, adoptive transfers of spleen cells or LAK cells derived from either Cmv-1− (C57BL/6) or Cmv-1+ (129) mice significantly lowered splenic MCMV titers in infected C57BL/6 sucking mice. Furthermore, perforin 0/0 spleen cells or LAK cells regulated MCMV in the spleens of the sucking mice, indicating that the regulation of MCMV by the transferred cells is perforin-independent (Table III). We and other groups have previously shown that the regulation of MCMV in the liver by NK cells in adult mice requires IFN-γ (12, 30). This mechanism is also used by the transferred cells in sucking mice, as normal C57BL/6 spleen cells, when transferred into IFN-γ R0/0 sucking mice, failed to regulate MCMV synthesis in the spleen. These results suggest that in the sucking mouse model the regulation of MCMV does not follow the convention seen in adult mice. Adoptively transferred cells, unlike the NK cells in the adult mouse model, used a perforin-independent, IFN-γ-dependent mechanism to control MCMV in the spleens of sucking mice. The adoptively transferred Ly49 NK cell subsets may have controlled MCMV via an IFN-γ-dependent mechanism.

It may not be surprising that NK cells use different mechanisms to control MCMV in the spleen of different mouse models. In the sucking mouse, both the effector cells and the pathogen are introduced into the peritoneal cavity. As the effector cells are present in the sucking mice before the introduction of the virus, it is possible that the control of MCMV may have occurred within the peritoneal cavity, thereby blocking the migration of the virus into the spleen. Early work by Bukowski et al. had shown that cultured purified Lyt2+ (CD8+) LAK cells, as well as a T cell clone with NK cell-like activity, when adoptively transferred into the sucking mice protected the mice from MCMV infection (6, 7). These non-NK cells are likely to have secreted IFN-γ and protected mice by that mechanism. Although there was a change of the tested Ly49 NK cell subsets in adult mice during MCMV infection and although individual
Ly49 NK cell subsets could control MCMV synthesis in suckling mice (probably by an IFN-γ mechanism), in vivo depletion of any one of the four tested Ly49 subsets did not affect the ability of the NK cells to control MCMV in adult spleen or liver. To date, there are nine cloned Ly49 NK cell receptors, but there are only Abs available to five of the receptors. The anti-Ly49D Ab (12A8) used in the above in vivo depletion and adoptive transfer studies is cross-reactive with the Ly49A NK cell receptor such that the depletion of Ly49D-bearing cells will also cause the depletion of Ly49A⁺ cells (20). Depleting cells with anti-Ly49G2 (4D11) Abs will not only deplete the Ly49G2⁺ cells but will also inadvertently deplete some Ly49A⁺ NK cells (33). This is because ∼1% of all NK cells express both Ly49A and Ly49G2 NK cell receptors (33). However, the cross-reactivity of anti-Ly49D Ab and the coexpression of Ly49A and Ly49G2 on some NK cells should not affect our understanding of the function of Ly49A subset. Depletion of the Ly49A NK cell subset or the adoptive transfer of the Ly49A⁺ or Ly49A⁻ LAK cells alone did not affect the ability of the NK cells to control MCMV in vivo.

Recently, the anti-Ly49C Ab (SW5E6) used in our analyses was found to bind not only with Ly49C but also with the Ly49I NK cell receptor. These data also suggest that in hybrid resistance, it is the Ly49C⁺/I⁺ NK cells and not the Ly49C⁺ NK cells that mediate the rejection of H-2b bone marrow cells (34). Nonetheless, results from the in vivo depletion of Ly49C⁻ and Ly49I-bearing cells (using SW5E6, Table II), or the adoptive transfer of Ly49C⁺, Ly49I⁺ or Ly49C⁻, Ly49I⁻ LAK cells (Table II) again strongly indicate that the absence or the presence of a particular Ly49 NK cell subset does not affect the ability of NK cells to control MCMV.

The Ly49D and Ly49H NK cell receptors are the only members of the Ly49 multigene family to date that do not contain the immunoreceptor tyrosine-based inhibitory motif (ITIM) motif in its cytoplasmic tail (20). Ly49D is a putative NK cell receptor, and Ly49D-bearing NK cells have been shown to have the ability to lyse tumor cells and ConA blasts of different H-2 haplotypes and mediate reverse antibody-dependent cell-mediated cytotoxicity (ADCC) through anti-Ly49D Ab on FcγR⁺ target cells (20). However, depleting the Ly49D⁺ NK cell subset or transferring Ly49D⁺ NKI.1⁺ LAK cells into MCMV-infected suckling mice did not affect the NK cells’ ability to control MCMV (Table II).

At first glance, these results suggest that the interaction of Ly49 molecules with their class I MHC ligands expressed on infected cells are probably not involved in the anti-viral activity of NK cells. In this regard, the results with adoptive transfer of various Ly49⁺ subset are of particular relevance. For example, the 5E6⁺ subset receives negative signals from H2Kb, therefore, in uninfected C57BL/6 mice this subset would be prevented from reacting against H2Kb-expressing (self) cells. Following viral infection, MCMV-associated peptides presumably displace the H2Kb-associated self-peptides, and such cells might be expected to be killed because they fail to deliver a negative signal to 5E6⁺ NK cell subset. This would explain the ability of 5E6⁺ subset to offer partial protection against MCMV infection. However, in the reported experiments, every subset, including Ly49A, D, and G2, which do not express known H2b receptors, protected as well as the 5E6⁺ subset. These data are not easily reconciled with the role for Ly49 molecules in the antiviral activity of NK subsets. However, it is

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Group</th>
<th>Log₁₀ PFU/Organ ± SD⁷</th>
<th>Splenic MCMV PFU</th>
<th>Liver MCMV PFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MCMV-infected control</td>
<td>&lt;2.4 ± 0.1</td>
<td>4.4 ± 0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MCMV-infected + anti-NK1.1</td>
<td>3.9 ± 0.3</td>
<td>4.8 ± 0.1</td>
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</tr>
<tr>
<td></td>
<td>MCMV-infected + anti-Ly49A</td>
<td>&lt;2.4 ± 0.1</td>
<td>4.3 ± 0.1</td>
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<tr>
<td>2</td>
<td>MCMV-infected control</td>
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<td>5.2 ± 0.1</td>
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</tr>
<tr>
<td></td>
<td>MCMV-infected + anti-NK1.1</td>
<td>4.0 ± 0.1</td>
<td>5.8 ± 0.1</td>
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<td>2.5 ± 0.3</td>
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<tr>
<td>3</td>
<td>MCMV-infected control</td>
<td>&lt;1.3 ± 0.0</td>
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</tr>
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<tr>
<td></td>
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<td>1.5 ± 0.2</td>
<td>3.6 ± 0.3</td>
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<td></td>
<td>MCMV-infected + anti-Ly49G2</td>
<td>1.6 ± 0.7</td>
<td>3.8 ± 0.1</td>
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<tr>
<td>4</td>
<td>MCMV-infected control</td>
<td>2.5 ± 0.1</td>
<td>4.1 ± 0.1</td>
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<tr>
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<td>MCMV-infected + anti-Ly49C + anti-Ly49G2</td>
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</tr>
<tr>
<td>5</td>
<td>MCMV-infected control</td>
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<td>6</td>
<td>MCMV-infected control</td>
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<td>4.3 ± 0.3</td>
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<tr>
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<tr>
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<td>MCMV-infected + anti-Ly49C + anti-Ly49D + anti-Ly49G2</td>
<td>2.5 ± 0.3</td>
<td>4.3 ± 0.3</td>
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</tbody>
</table>

⁷ Age-matched C57BL/6 mice were infected i.p. with 10⁶ MCMV PFU per mouse. Anti-NK1.1, anti-Ly49A, anti-Ly49C, and anti-Ly49G2 were given i.v. 1 day prior to infection. Anti-Ly49D was given i.v. at day 2 and day 1 prior to infection.

* Composed with the infected control, anti-NK cell treatment resulted in a p < 0.005.

* Compared with the infected control, anti-NK cell treatment resulted in a p < 0.05.

* Compared with the infected control, anti-NK cell treatment resulted in a p > 0.5.

* Compared with the infected control, anti-NK cell treatment resulted in a p ≤ 0.5.
conceivable that each of the Ly49 subsets expresses other unknown Ly49 or non-Ly49 receptors that normally inhibit lysis of unmodified cells, but allow the lysis of virus-infected cells due to alterations in class I molecules. Such receptors would have to be expressed on all NK subsets, and may be far less class I-specific than Ly49 molecules. The recently described CD94 molecules would be candidates for such non-Ly49 class I MHC receptors. This receptor is expressed on all human NK cells and seems to have a broad reactivity with MHC molecules, and it is possible that mouse NK cells may also express such molecules that are used to detect virus-infected cells.

References