m144, a Murine Cytomegalovirus (MCMV)-encoded Major Histocompatibility Complex Class I Homologue, Confers Tumor Resistance to Natural Killer Cell–mediated Rejection

By Erika Cretney,* Mariapia A. Degli-Esposti,‡ Eloise H. Densley,‡ Helen E. Farrell,§ Nick J. Davis-Poynter,§ and Mark J. Smyth*

*From the *Cellular Immunity Laboratory, The Austin Research Institute, Melbourne 3084, Victoria, Australia; the ‡Department of Microbiology, The University of Western Australia, Perth 6009, Western Australia, Australia; and the §Centre for Preventative Medicine, Suffolk CB8 7UU, United Kingdom*

Summary

Until now, it has been unclear whether murine cytomegalovirus (MCMV)-encoded protein m144 directly regulates natural killer (NK) cell effector function and whether the effects of m144 are only strictly evident in the context of MCMV infection. We have generated clones of the transporter associated with antigen processing (TAP)-2–deficient RMA-S T lymphoma cell line and its parent cell line, RMA, that stably express significant and equivalent levels of m144. In vivo NK cell–mediated rejection of RMA-S-m144 lymphomas was reduced compared with rejection of parental or mock-transfected RMA-S clones, indicating the ability of m144 to regulate NK cell–mediated responses in vivo. Significantly, the accumulation of NK cells in the peritoneum was reduced in mice challenged with RMA-S-m144, as was the lytic activity of NK cells recovered from the peritoneum. Expression of m144 on RMA-S cells also conferred resistance to cytotoxicity mediated in vitro by interleukin 2–activated adherent spleen NK cells. In summary, the data demonstrate that m144 confers some protection from NK cell effector function mediated in the absence of target cell class I expression, but that in vivo the major effect of m144 is to regulate NK cell accumulation and activation at the site of immune challenge.

Key words: mouse • tumor immunity • natural killer cells • cytotoxicity • cytomegalovirus

Natural killer (NK) cells play an important role in the early defense against herpesvirus infection (1–3). In humans, patients with NK cell deficiencies demonstrate increased susceptibility to herpesvirus infections, such as human CMV (HCMV)[1] (4). The mechanisms of NK cell–mediated protection against herpesvirus infection have been well characterized in the murine CMV (MCMV) model; NK-deficient animals are highly susceptible to MCMV infection, and in vivo transfer of NK cells confers protection. In addition, the autosomal dominant non-H gene, designated Cmv-1, regulates MCMV replication through the action of NK cells (1).

The ability of herpesviruses to persist lifelong in infected individuals suggests that these viruses possess mechanisms for evading the host protective immune response (5–7). Indeed, recent studies have characterized several herpesvirus immune-evasion strategies, in particular the downregulation of host MHC class I expression (6, 7). This process enables evasion of CTL-mediated immune responses, but potentially would leave infected cells more vulnerable to NK cell–mediated lysis.

MCMV and HCMV genomes each encode a homologue of MHC class I heavy chains, designated m144 and UL18, respectively (8–10). Although both m144 and UL18 bind β2-microglobulin like their cellular counterpart, only UL18 has been shown to mimic class I in the binding of endogenous peptides (9). The inability of m144 to bind endogenous peptides may be due to a deletion within the m144 α-2 domain encoding the predicted peptide-binding groove (10). We have previously demonstrated that a recombinant MCMV with a disrupted m144 open reading frame (Δm144) has severely restricted replication during acute infection compared with wild-type MCMV (11). Depletion of lymphocyte subsets in infected mice showed that Δm144 was cleared in vivo in an NK cell–dependent manner (11), suggesting that m144 may be regulating NK cell activation and/or effector responses.

It has been hypothesized that m144 mimics the host class I molecules and thereby acts as a decoy for NK cells by engaging NK cell inhibitory receptors, thus protecting in-

[1]*Abbreviations used in this paper:* B6, C57BL/6; HCMV, human CMV; LIR, leukocyte Ig-like receptor; MCMV, murine CMV; P^0, perforin-deficient; RAG, recombination activating gene; TAP, transporter associated with antigen processing.

fected cells from NK cell–mediated lysis (9, 11, 12). A nonclassical MHC class I molecule, HLA-E, has been shown to inhibit NK cell–mediated cytotoxicity by interaction with the CD94/NKG2A inhibitory receptor (13, 14), while another, HLA-G, was demonstrated to inhibit NK cell–mediated lysis by interaction with KIR2DL4 (15). More recently, murine CD1.1, a nonpolymorphic nonclassical MHC class I–like molecule, has been shown to negatively regulate NK cell activity (16).

To date, a direct interaction between m144 and NK cell surface ligands has not been established, nor has the m144 ligand been isolated. Expression of m144 in the human cell line Raji offered these targets some protection from xenogeneic antibody-dependent cellular cytotoxicity (17). To address whether m144 could directly inhibit NK cell effector function and/or NK cell activation in a syngeneic model, we have transfected m144 into a mouse class I–deficient lymphoma. Here we demonstrate that m144 can confer protection to syngeneic lymphoma cells from activated NK cell–mediated lysis in vitro and in vivo. Furthermore, we provide the first evidence that the accumulation and activation of NK cells in vivo can also be compromised by cellular expression of m144.

Materials and Methods

Mice. Inbred C57BL/6 (B6) and BALB/c mice were purchased from The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia. B6 perforin-deficient (B6.P^0) mice (18) were obtained from Dr. Guna Karupiah, John Curtin School of Medical Research, Canberra, Australia. B6 recombination activating gene (RAG)-1–deficient (B6.RAG-1^0) mice were provided by Dr. Lynn Corcoran, The Walter and Eliza Hall Institute of Medical Research. Mice 4–6 wk of age were used. All experiments were performed according to animal experimental ethics committee guidelines.

Cell Culture and Reagents. The YAC-1 (H-2^a) and RMA (H-2^b) lymphoma cell lines and RMA-S (H-2^b) mutant lymphoma (derived from the Rauscher virus–induced murine cell line RBL-5 and defective for peptide loading of MHC class I molecules) were grown in RPMI medium supplemented with 10% (vol/vol) FCS, 2 mM glutamine,100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO BRL). RMA-S/RMA-m144 transfectants were grown in complete RPMI medium with 800 μg/ml G418 (GIBCO BRL). Recombinant human IL-2 was provided by Chiron Corp. Adherent IL-2–activated NK cells were generated by culturing splenocytes in complete RPMI medium with 1,000 U/ml IL-2 for a minimum of 7 d. These populations were generally >60% NK1.1$^+$ (data not shown). Spleens from B6.RAG-1^0 mice were additionally used as a source of NK cell effector cells. In some experiments, NK cell cultures were derived by harvesting spleen cells from B6 mice and depleting Thy-1.2$^+$ cells using anti–Thy-1.2 mAb (rat IgG2a) and C′ (rabbit 1:30 dilution) before culture in IL-2 as described (19). Mouse CD8$^+$ CTLs reactive with the human papilloma virus 16 E7 peptide (RAHYNIVTF) were generated as described previously (20).

Stable Transfection of m144 into Lymphoma Cell Lines. A pCDNA-m144 construct encoding full-length COOH-terminal c-myc–tagged MCMV m144 was constructed. RMA or RMA-S cells were transfected with CsCl-purified pCDNA-m144-tag vector DNA by electroporation (250 V, 960 μF; BioRad Gene Pulser). Cells (5×10^6) in RPMI were added to 25 μg of vector DNA in 4-mm cuvettes. After electroporation, cells were added to supplemented RPMI and aliquoted into a 96-well plate. 2 d later, the medium was removed from the cells and new medium with 800 μg/ml G418 was added to select for transfected cells. After 1 wk in selection medium, cells were replenished with fresh selection medium and viable clones were tested for m144 cell surface expression by flow cytometry and Western analysis.

^{51}Cr-release Assays. Direct cytotoxicity of resting and IL-2–activated spleen NK cells or CTLs reactive with the E7 peptide was assessed by 4-h ^{51}Cr-release assays against labeled target cells. For CTL assays, temperature-induced RMA-S or RMA-S-m144 cells were incubated at 25°C for 24 h and then with E7$^{49–57}$ or control chicken OVA$^{257–264}$ (SIINFEKL) peptide for 2–4 h at 33°C as described (20). In some experiments, ^{51}Cr-labeled RMA-S or RMA-S-m144 cells and unlabeled RMA-S-m144 target cells (at various E/T ratios) were added to IL-2–activated spleen NK cells. In all experiments, the spontaneous release of ^{51}Cr was determined by incubating the target cells (2×10^4) with medium alone, while the maximum release was determined by adding SDS at a final concentration of 5%. The percent specific lysis was calculated as follows: 100 × [(experimental release − spontaneous release)/(maximum release − spontaneous release)]. Each experiment was performed at least twice using triplicate samples.

Flow Cytometry. RMA-S/RMA and their transfectants were phenotyped by flow cytometry using a FACStar$^{PLUS™}$ (Becton Dickinson). The following mAbs were provided as indicated: anti–H-2K^{b}D^b (Dr. P. Xing, The Austin Research Institute); anti-m144 (15C6; Dr. T. Chapman, California Institute of Technology, Pasadena, CA); and anti–Ly-49A (YE132), C (5E6), D (4E5), G2 (4D11), and I (5E6) (Dr. J. Ortaldo, Frederick Cancer Research and Development Center, Frederick, MD). Anti-NK1.1 (PK136), anti–ICAM-1 (3E2), anti-CD1d (1B1), anti-CD11a (M17/4), anti-CD80 (1G10), and anti-CD86 (GL1) mAbs were purchased from PharMingen. Anti–mouse CD3 (29B) was purchased from Sigma Chemical Co. Cells were incubated at 4°C (30 min) with primary antibody, washed, and stained with FITC anti–mouse Ig F(ab′)$_2$ purchased from Silenus.

Western Analysis. MCMV m144 expression was measured by Western analysis. Cells (2×10^5) were lysed in 70 μl of ice-cold NP-40 lysis buffer (25 mM Hepes, pH 7, 250 mM NaCl, 2.5 mM EDTA, 0.1% NP-40, 0.5 mM dithiothreitol, and 2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride [AEBSF]) for 30 min at 4°C. Insoluble material was removed by centrifugation at 4°C. The proteins were separated on 10% SDS-PAGE, blotted onto Immobilon-P (Millipore Corp.), and visualized by ECL chemiluminescence (Nycomed Amersham plc).

Tumor Growth. Groups of five B6 or B6.P^0 mice were injected with 10^1–10^5 RMA-S, RMA, RMA-S-m144, RMA-m144, or RMA-S-neo cells intraperitoneally and monitored daily for tumor ascites development indicated by swelling of the abdomen or loss of >20% body weight. Mice were culled when these obvious signs of tumor growth were noted, and those surviving beyond 100 d were deemed tumor free.

NK Cell Accumulation in the Peritoneum after Tumor Challenge. The number of NK1.1$^+$ cells accumulating in the peritoneum was evaluated in B6 mice that had received intraperitoneal PBS (0.2 ml), RMA-S, or RMA-S-m144 tumor cells (10^3) as described previously (21). After 72 h, mice were killed by CO_2 asphyxiation and their peritoneal cavities were flushed with 0.5 ml complete RPMI and aspirated with a syringe. The percentage of NK cells recovered was determined by staining with FITC-labeled

anti-NK1.1 (PK136; relative to a negative control), and an absolute number of NK1.1$^+$ cells was calculated on the basis of simultaneously obtained cell count. The NK cell cytotoxicity mediated by NK cells recovered from the peritoneum was measured against YAC-1 target cells after culturing effector cells overnight with IL-2 (1,000 U/ml). The results were recorded as $LU_{20}/10^7$ cells, where one LU is the number of effector cells required to lyse 20% of the target cells.

Results

Expression of m144 in Murine RMA and RMA-S T Lymphoma Cells. The murine T cell lymphoma RMA and its class I–deficient mutant RMA-S were transfected with m144-tag cDNA, and several transfectants expressing m144 were obtained. Flow cytometric analysis confirmed that equivalent levels of surface m144 expression were obtained in both RMA and RMA-S cell lines (Fig. 1, A and B). As expected, RMA cells expressed high levels of class I, whereas the RMA-S cells expressed low, but nonfunctional, class I levels. Expression of m144 did not alter endogenous MHC class I levels, since parental cell lines and their m144 transfectants expressed similar levels of H-2^b (RMA-S, mean channel fluorescence [MCF] = 36.3 ± 5.1; RMA-S-m144, MCF = 38.3 ± 7.2; n = 8; Fig. 1, C and D). The expression of other cell surface molecules that regulate cell–cell interactions (e.g., CD1d, CD11a, and ICAM-1) were also shown to be the same in parental cells and their transfectants, while these cells did not express CD80 or CD86 (data not shown). Expression of m144 in the transfectants was further verified by Western analysis (Fig. 2 A) with the demonstration of an ~63-kD protein carrying ~18 kD of glycosylation that could be removed by tunicamycin treatment (1 μg/ml overnight). This treatment reduced the size of m144 to ~45 kD (Fig. 2 B). In all in vitro and in vivo experiments discussed below, several m144 clones were examined, and the results presented are representative.

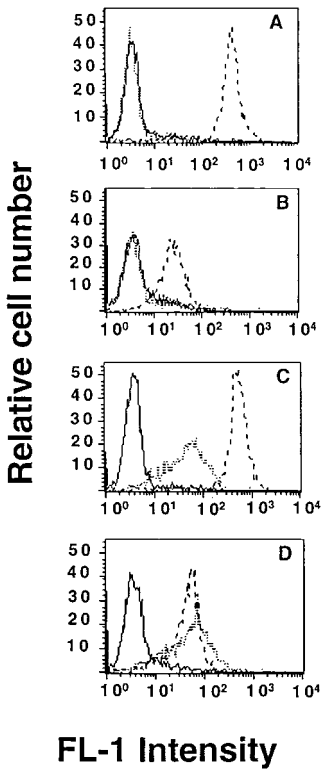


Figure 1. Surface expression of MCMV m144 and MHC class I (H-2^b) in parental and m144-transfected lymphomas. (A) RMA, (B) RMA-S, (C) RMA-m144, and (D) RMA-S-m144. Isotype control mAb (solid line), anti-m144 15C6 mAb (dotted line), and anti–H-2K^{b}D^b mAb (dashed line). Cells were incubated at 4°C (30 min) with primary antibody, washed, and stained with FITC anti–mouse Ig F(ab′)$_2$ before analysis by flow cytometry. These profiles were representative of more than five independent RMA-m144 and RMA-S-m144 clones.

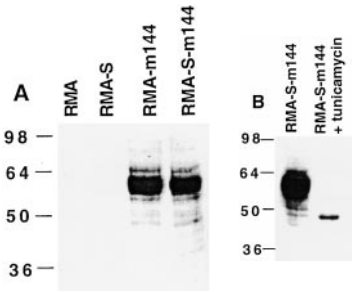


Figure 2. Western analysis of m144 expression in RMA-m144 and RMA-S-m144 transfectants. (A) Parental lymphoma cell lines (lanes 1 and 2) compared with RMA-m144 and RMA-S-m144 transfectants (lanes 3 and 4) when probed with anti-m144 15C6 mAb. The m144 protein migrated broadly at ~57–63 kD. (B) Tunicamycin treatment (1 μg/ml overnight) reduced the m144 migrating species to one band at ~45–47 kD. Proteins were separated by SDS-PAGE and detected by Western analysis as described in Materials and Methods. The positions of the protein standards are shown in kD. These profiles were representative of at least three independent RMA-m144 and RMA-S-m144 clones.

m144 Protection of Class I–deficient Lymphoma In Vivo. The class I–deficient lymphoma RMA-S is rejected from syngeneic mice in a perforin-dependent manner by NK cells (21, 22). To investigate whether m144 can confer resistance to NK cell–mediated rejection, B6 mice were injected intraperitoneally with increasing doses (10^1–10^5) of RMA-S, RMA-S-m144, or RMA-S-neo (transfectant that lost m144 expression but retained neo resistance) lymphoma cells. Compared with RMA-S or RMA-S-neo cells, at least 10-fold less RMA-S-m144 cells were required to establish tumor growth, suggesting that m144 confers resistance to NK cell–mediated tumor rejection (Fig. 3 A). For example, all B6 mice injected with 10^3 RMA-S-m144 cells died within 55 d, whereas RMA-S– or RMA-S-neo–injected mice remained tumor free. Recovery and flow cytometry evaluation of lymphoma ascites from mice that succumbed to RMA-S-m144 inoculation indicated that m144-expressing tumor cells had retained surface expression of m144 in five of six mice examined, and all of these tumors still expressed m144 by Western analysis (data not shown). To demonstrate that m144 was specifically protecting the lymphoma cells from perforin-mediated cytotoxicity, B6.P^0 mice were examined for their ability to reject lymphoma. In B6.P^0 mice, regardless of the dose of tumor cells administered, the rejection of RMA-S, RMA-S-neo, and RMA-S-m144 lymphoma cells was equivalent (Fig. 3 B). The specificity of the m144 protection against NK cell–mediated responses was demonstrated by the equivalent rejection of class I–expressing RMA and RMA-m144 cells in B6 and B6.P^0 mice (data not shown). In summary, these in vivo tumor rejection assays indicated for the first time that MCMV m144 has the potential to regulate a cellular immune response even when expressed in isolation from other MCMV genes.

Defective Accumulation and Activation of NK Cells in Response to RMA-S-m144 Inoculation. From previous studies (21), it was known that NK cell accumulation in the peritoneum

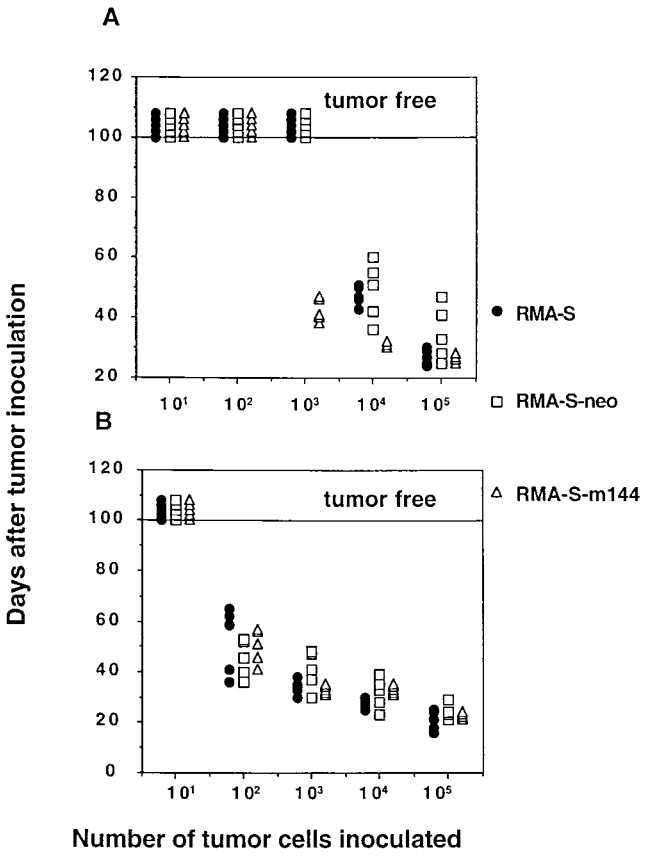


Figure 3. m144 expression reduces the elimination of intraperitoneally administered MHC class I–deficient syngeneic lymphomas. (A) B6 mice were injected intraperitoneally with 10^1–10^5 RMA-S, RMA-S-m144, or RMA-S-neo tumor cells in 0.2 ml PBS. Mice were observed daily for tumor growth up to 100 d by monitoring body weight and development of ascites in mice. Individual mice are represented by each symbol and are representative of three independent experiments. These data include mice inoculated with one of four different RMA-S-m144–transfected clones. (B) B6.P^0 mice were inoculated with 10^1–10^5 tumor cells as above.

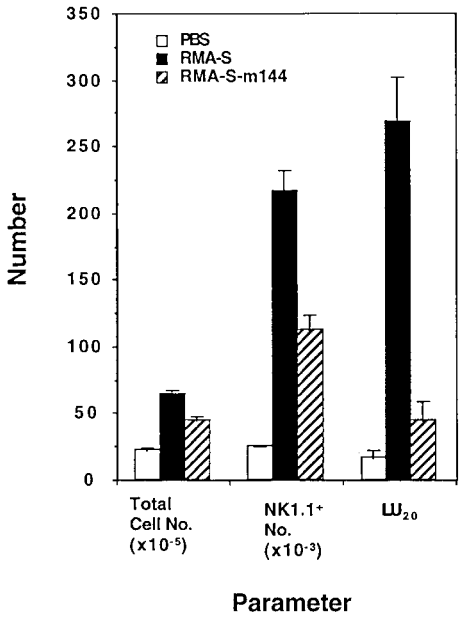


Figure 4. The lytic activity and accumulation of NK cells are reduced in mice inoculated with RMA-S-m144 lymphoma cells. Mice were injected intraperitoneally with PBS (white bars), RMA-S (10^3; black bars), or RMA-S-m144 (10^3; striped bars) cells. 72 h later, the peritoneal lavages were collected, and we recorded (a) total leukocyte numbers ($\times 10^{-5}$) and (b) total NK1.1$^+$ cell numbers ($\times 10^{-3}$). The cells collected from the lavage were incubated with 1,000 U/ml IL-2 overnight, and the lytic activity of NK cells in the culture was determined by ^{51}Cr-release assay against labeled YAC-1 targets and recorded as $LU_{20}/10^7$ where 2×10^4 YAC-1 target cells were added per well at E/T ratios ranging from 200:1 to 1:1.

was maximal after ~3 d in an effective response regardless of the number of RMA-S tumor cells inoculated. To further investigate how MCMV m144 might modulate NK cell–mediated clearance of class I–deficient lymphomas, B6 mice were inoculated intraperitoneally with PBS, RMA-S (10^3), or RMA-S-m144 (10^3) cells. 3 d later, the total number of leukocytes and NK1.1$^+$ cells accumulated in the peritoneum was assessed (Fig. 4). Interestingly, although the total number of leukocytes was increased in mice inoculated with either tumor, there was comparatively a 30% reduction in RMA-S-m144–inoculated mice. Even more strikingly, RMA-S-m144–inoculated mice had a twofold lower number of NK1.1$^+$ cells accumulated in the peritoneum compared with RMA-S–inoculated mice, suggesting that expression of m144 on RMA-S lymphoma cells reduced NK cell accumulation at the tumor site. Next we examined the NK cell lytic capacity of the recovered peritoneal cells (cultured in IL-2 overnight) against YAC-1 targets. Clearly, the peritoneal cells from RMA-S-m144–inoculated mice were sixfold less cytotoxic than those from RMA-S–inoculated mice (Fig. 4). Even when taking into account the twofold decrease in peritoneal NK cells from RMA-S-m144–inoculated mice, on a per NK cell basis the lytic activity of NK cells in mice inoculated with RMA-S-m144 was reduced approximately threefold compared with mice challenged with RMA-S. It is not clear why the lytic potential of NK cells from RMA-S-m144–inoculated mice was reduced nor why IL-2 stimulation overnight was insufficient to restore cytotoxicity. Further efforts to purify peritoneal NK cells from tumor-inoculated mice and activate their lytic program may reveal whether NK cell activation is suboptimal in the presence of m144.

MCMV m144 Protected RMA-S Target Cells from Syngeneic NK Cell–mediated Cytotoxicity. Since it had previously been suggested that m144 could directly inhibit NK cell–mediated cytotoxicity in vitro (17), we evaluated the sensitivity of RMA-S-m144 and RMA-m144 target cells to cytolysis induced by syngeneic B6 NK cells. In agreement with Ljunggren et al. (23), resting and IL-2–activated spleen NK cells lysed class I–deficient RMA-S cells efficiently, whereas class I–expressing RMA cells were only lysed to a minimal extent (Fig. 5, A and B). RMA-S-m144 transfectants were relatively less sensitive (~50%) than RMA-S cells to IL-2–activated NK cells, whereas RMA-m144 and RMA were lysed to the same extent (Fig. 5 B). Surprisingly, RMA-S-m144 transfectants were not more resistant to lysis mediated by resting NK cells (Fig. 5 A), suggesting that activation of the spleen NK cell population

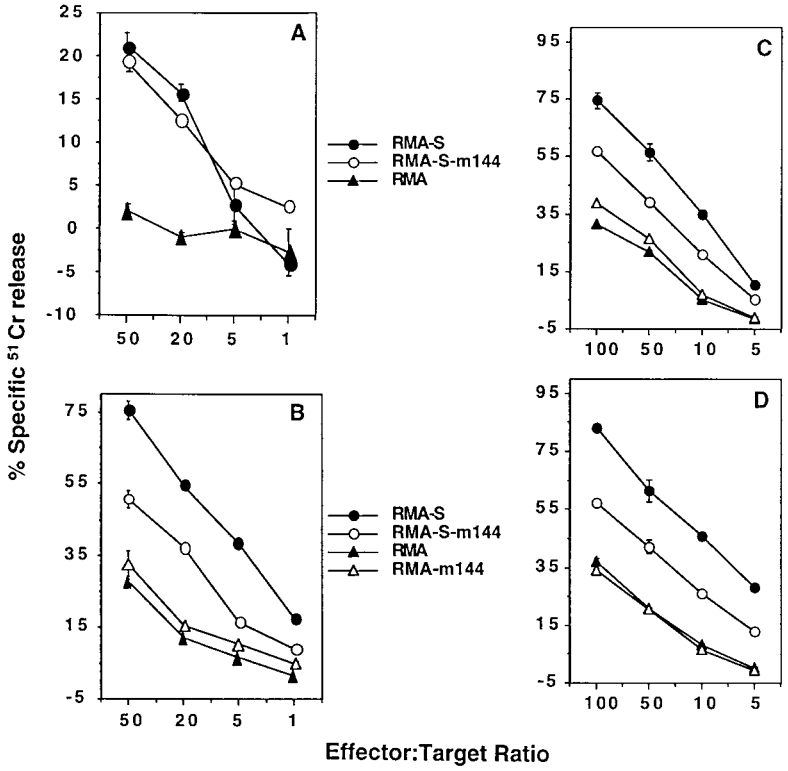


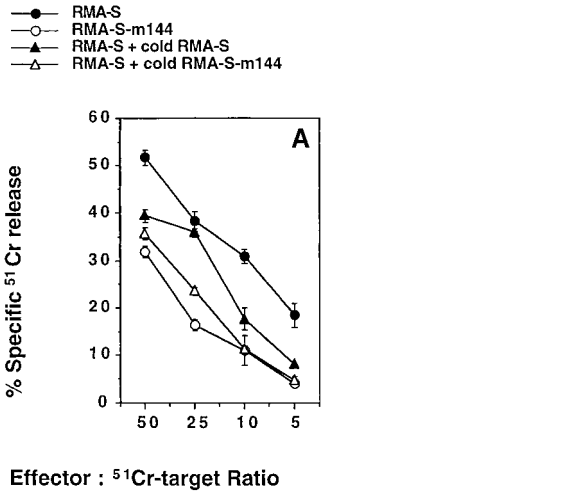
Figure 5. MCMV m144 inhibits the cytotoxicity mediated by IL-2–activated NK cells. RMA-S (filled circles), RMA-S-m144 (open circles), RMA (filled triangles), and RMA-m144 (open triangles) were labeled with ^{51}Cr and used as targets in a standard 4-h ^{51}Cr-release assay. Effectors were generated from (A) whole resting spleen cells from B6 mice, (B) IL-2 (1,000 U/ml)–activated (7 d) spleen cells from B6 mice, (C) anti–Thy-1 depleted and IL-2–activated (7 d) spleen cells from B6 mice, and (D) IL-2–activated (7 d) spleen cells from B6.RAG-1^0 mice. E/T ratios were as shown with 2×10^4 labeled target cells/well. The spontaneous release of ^{51}Cr was always <15%, and all tests were performed in triplicate. These assays were representative of at least three different RMA-m144– and RMA-S-m144–transfected clones and were repeated for A–D in two to three experiments for each clone.

was critical for m144-mediated protection and that activated, rather than unstimulated, NK cells may express the m144 counterstructure. These data suggested that NK lysis of class I–expressing target cells was not regulated by target cell m144 expression. To reduce potential T-LAK–mediated lysis in spleen NK cell cultures, spleen cells were initially depleted of Thy-1.2$^+$ T cells before IL-2 culture. Cultures containing >95% IL-2–activated NK1.1$^+$ cells were more lytic. Using this system, we also demonstrated that m144 conferred some protection to RMA-S target cells (Fig. 5 C). Potential nonspecific cytotoxicity by T cells and NKT cells was also avoided by using IL-2–activated NK cell cultures from B6.RAG-1^0 mice as effector cells. Once again, m144 protected RMA-S-m144 target cells from lysis mediated by IL-2–activated NK cells. Class I–expressing target cells were not protected (Fig. 5 D).

Since mAbs that inhibit m144 function have not been described and, in agreement with a previous study (17), the 15C6 anti-m144 mAb did not affect lysis of RMA-S-m144 target cells (data not shown), we examined the specificity of m144 protection by cold target inhibition studies. Two different assays were performed: in the first assay, equal numbers of cold and ^{51}Cr-labeled target cells were added to effectors at a variety of E/T ratios (Fig. 6 A); in the second assay, the number of labeled target cells was kept constant and the competitor cold target cell number was varied (Fig. 6 B). In both assays, the lysis of ^{51}Cr-labeled RMA-S target cells was more effectively inhibited by the presence of cold targets expressing m144 (Fig. 6, A and B). Based on the similarity of MCMV m144 to MHC class I molecules, it was possible that the cytoplasmic tail of m144 was affecting the sensitivity of RMA-S target cells to cell death or simply that the clones selected for m144 expression were also more resistant to cell death. To test this possibility, RMA-S and RMA-S-m144 cells were pulsed with human papilloma virus E7$^{49–57}$ peptide and exposed to cytotoxic CD8$^+$ T cells specific for this peptide (Fig. 7). Pulsed RMA-S and RMA-S-m144 target cells were equally sensitive to E7-specific CTL lysis, indicating that class I molecules could be functional when loaded, irrespective of m144 expression, and that m144 does not protect target cells from all forms of cell-mediated death. Furthermore, in effector-free systems, RMA-S and RMA-S-m144 target cells were equally sensitive to soluble FasL or perforin/granzyme B, the major effector mechanisms used by CTLs and NK cells (data not shown). Although these in vitro studies have demonstrated a direct protective effect of m144 on NK cell–mediated lysis, it is likely that a more significant role of m144 involves the regulation of NK cell activation and accumulation which in our studies correlated with resistance to tumor rejection.

Discussion

In this study, MCMV m144 was transfected into the murine T lymphoma cell line RMA and its class I–deficient derivative RMA-S. We have demonstrated that in vitro m144 can directly inhibit cytolysis mediated by IL-2–activated NK cells, but this protection was only partial and restricted to the activity of IL-2–activated NK cells against class I–deficient target cells. More importantly, m144 protected RMA-S lymphoma cells from NK cell–mediated re-



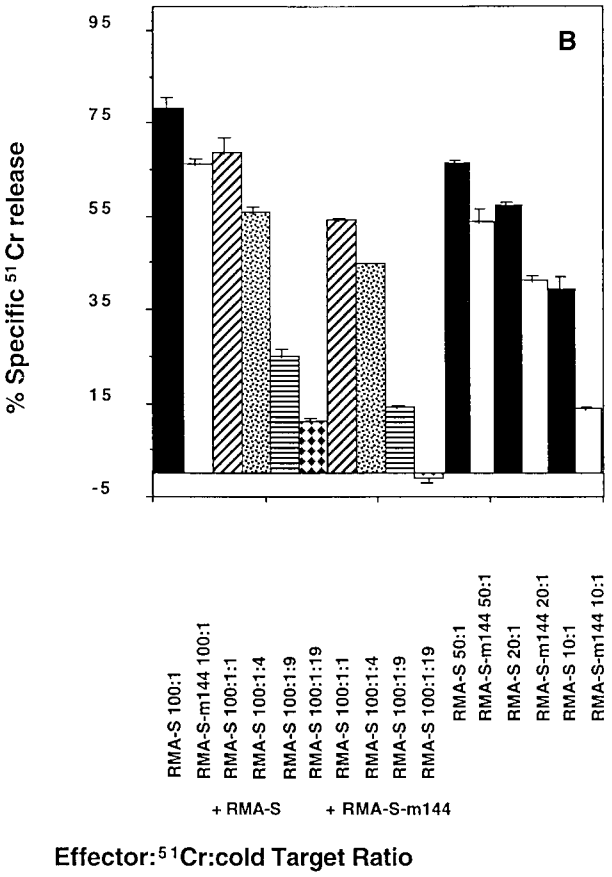


Figure 6. Competitive inhibition of NK cell–mediated cytotoxicity. RMA-S (filled circles) and RMA-S-m144 (open circles) were labeled with ^{51}Cr and used as targets in a standard 4-h ^{51}Cr-release assay. Effectors were generated from IL-2 (1,000 U/ml)–activated (7 d) spleen cells from B6 mice. In A, an equal number of ^{51}Cr-labeled RMA-S targets and competitor unlabeled RMA-S (filled triangles) or RMA-S-m144 (open triangles) targets were added (ratio relative to number of ^{51}Cr-labeled targets). In B, an increasing number of cold RMA-S or RMA-S-m144 target cells were added as indicated to ^{51}Cr-labeled RMA-S target cells fixed at a ratio of 100:1 (effector/^{51}Cr-labeled target/unlabeled target ratio shown). The spontaneous release of ^{51}Cr was always <15%, and all tests were performed in triplicate. These results are representative of two experiments.

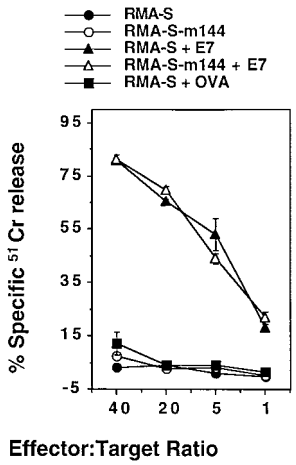


Figure 7. CTL-mediated cytotoxicity of lymphoma cells is unaffected by m144 expression. RMA-S (filled circles) and RMA-S-m144 (open circles) were labeled with ^{51}Cr and used as targets in a standard 4-h ^{51}Cr-release assay. In some wells, RMA-S or RMA-S-m144 cells had been prepulsed with $E7^{49–57}$ or $OVA^{257–264}$ peptide. Effector cells were generated from the spleens of E7-immunized B6 mice as described previously (reference 21). E/T ratios were as shown with 2×10^4 labeled target cells/well. The spontaneous release of ^{51}Cr was always <15%, and all tests were performed in triplicate. These results are representative of at least two different RMA-S-m144 clones.

jection in vivo, a process that requires NK cell recruitment, activation, and effector function. These data are the first to demonstrate that m144 can, in isolation from other MCMV proteins, regulate NK cell–mediated immune responses and support our previous work that suggested that m144 can prevent NK cell–mediated clearance of MCMV in vivo (11).

The dramatic effects of m144 expression on NK cell accumulation and rejection of RMA-S lymphoma cells in vivo are unlikely to be explained by the comparatively minor protective effect of m144 against NK cell–mediated lysis demonstrated in vitro. We have shown that m144 protected RMA-S lymphomas from NK cell–mediated rejection in vivo by affecting the tumor rejection process at several levels. m144 reduced the number of total leukocytes and NK cells (by two- to threefold) recruited to the peritoneum in response to tumor challenge. In addition, the markedly reduced lytic capacity of NK cells recovered from the peritoneum of RMA-S-m144–challenged mice suggests that the activation of NK cells at the tumor site was also decreased when m144 is expressed. Previous studies of MCMV infection in the liver have indicated the importance of IL-12 for NK cell–mediated IFN-γ production (2) and macrophage inflammatory protein 1α (MIP-1α) for NK cell infiltration (24). The key initial events involved in recruitment of NK cells and other leukocytes in response to peritoneal tumor challenge remain unclear. Previous experiments using the RMA-S lymphoma have demonstrated that T cells, B cells, F4/80$^+$ cells, and IL-12 are not critical in effective NK cell–mediated rejection, but that TNF is important for NK cells to effectively accumulate in response to RMA-S tumor challenge in the peritoneum (21). Therefore, we favor the possibility that a local interaction between APCs, NK cells, and stromal elements leads to the development of a cytokine/chemokine network that regulates the innate response to tumor. This model would predict that m144 may interact with an inhibitory receptor on local APCs, ultimately reducing APC cytokine/chemokine secretion and therefore NK cell activation. Leong et al. (25) have suggested that the human CMV class I homologue, UL18, might function in such a manner, a suggestion supported by the ability of UL18-Fc fusion proteins to bind

leukocyte Ig-like receptor (LIR-1) expressed predominantly on B cells and monocytes and a small population of NK cells (26). It has also been noted that the subpopulation with the highest LIR-1 expression is a major source of IFN-α, a potent NK cell stimulus (27). Although studies with the closest murine homologue of LIR-1 have not revealed binding to m144 (Chapman, T., unpublished data), we cannot exclude the possibility that m144 does interact with an inhibitory receptor on a cell that is normally the source of cytokines essential for NK cell activation. The observation that m144 did not protect RMA-m144 lymphoma cells from rejection indicates that m144 specifically regulates those immune responses critical for NK cell–mediated tumor rejection. m144 did not alter rejection of RMA-S tumor in B6.P^0 mice simply because NK cells exclusively use perforin to reject RMA-S lymphoma from the peritoneum (21), and thus NK cell responses to parental RMA-S are completely abrogated in B6.P^0 mice.

Our in vitro studies demonstrated that m144 was able to inhibit direct syngeneic NK cell–mediated cytotoxicity. Nevertheless, the level of protection afforded was never complete and was only observed in cultures where nonrestricted lysis of class I–expressing target cells was low. In some cultures using whole spleen cells activated with IL-2 as effectors, lysis of RMA cells was comparable to lysis of RMA-S cells, and m144 expression afforded no protection to RMA-S-m144 transfectants (data not shown). These data contrast with the complete protection mouse CD1.1 afforded RMA-S cells from A-LAK cytotoxicity (16). It is likely that the relative concentration of the appropriate ligand on the effector NK cells may explain these differences, since selected late-adhering A-LAK cultures (16) are more homogeneous than the IL-2–activated spleen cultures we have used. In this context, it is possible that only those NK cells expressing relatively high levels of the putative m144 ligand receive the m144 inhibitory signal. Alternatively, in the absence of data relating to the level of m144 expression on the surface of MCMV-infected cells, it is possible to postulate that the levels of m144 expression on RMA-S target cells may be too low to deliver inhibitory signals to all NK cells. Clearly however, m144 offered no protection from cytotoxicity mediated by unstimulated spleen NK cells, suggesting that the m144 ligand may be scarce or conformationally inactive on resting NK cells. Since m144 had no effect on the lysis of RMA cells, the m144 ligand may be absent on NK cells that are not inhibited by target cell class I expression. From preliminary studies, m144 is not delivering an apoptotic signal to activated NK cells (data not shown).

The TAP-2–deficient cell line RMA-S can present peptides derived from cytosolic proteins on classical MHC class I through a TAP-independent pathway (28). Thus, it could be argued that the protective effect seen in RMA-S-m144 is actually mediated by m144-derived peptides stabilizing normally thermally unstable D^b and K^b molecules expressed by RMA-S. Evidence against this argument is provided by the finding that no reproducible increase in cell surface expression of D^b and K^b was detected in RMA-S-m144 transfectants. Furthermore, given that murine class I Qa-1^b can present leader sequences from murine class Ia molecules and may bind inhibitory CD94/NKG2A heterodimers (29, 30), it is possible that m144 inhibition of NK cell–mediated lysis is actually mediated by Qa-1^b. However, the leader sequence of m144 does not contain the necessary motif to bind Qa-1^b. Alternatively, as RMA-S cells transfected with CD1d have been demonstrated to inhibit NK cell cytotoxicity (16), it may be argued that this molecule (which is normally expressed at low levels in RMA-S cells) may be involved in the protective effects of m144. However, expression of m144 on RMA-S cells did not appear to upregulate Cd1d expression, indicating that the protection conferred by m144 was CD1d independent.

In summary, MCMV m144 conferred some protection from NK cell effector function mediated in the absence of target cell class I expression, but in vivo the major effect of m144 was to regulate NK cell accumulation and activation at the site of tumor challenge. It remains to be determined whether inhibiting NK cell activation and accumulation is the principal role of m144 during viral infection. However, infections comparing wild-type and m144-deficient strains of MCMV suggested that the activation of NK cytolysis in situ was inhibited >10-fold in the presence of m144 (data not shown). The cytokine- and cytotoxicity-mediated mechanisms of virus clearance by NK cells have been shown to play different roles in different organs of the infected animal (31). It is possible that the m144 molecule may function during infection to inhibit both mechanisms of NK cell–mediated clearance of virus in vivo. Identification and characterization of the MCMV m144 ligand(s) and the leukocyte subsets that are regulated by m144 expression will greatly improve our understanding of MCMV evasion of host immune responses and will clarify the relationships between NK and other innate immune cells.

We thank Dr. Tara Chapman for the anti-m144 mAb, and Drs. Joe Trapani and Ricky Johnstone for helpful discussions.

M.J. Smyth and E. Cretney are currently supported by a National Health and Medical Research Council of Australia (NH&MRC) Research Fellowship and Project Grant, respectively. M.A. Degli-Esposti and E.H. Densley are supported by a Project Grant from the NH&MRC.

Address correspondence to Mark Smyth, Cellular Immunity Laboratory, The Austin Research Institute,

Studley Road, Heidelberg 3084, Victoria, Australia. Phone: 61-3-9287-0653; Fax: 61-3-9287-0600; E-mail: m.smyth@ari.unimelb.edu.au

Submitted: 26 April 1999 Revised: 2 June 1999 Accepted: 7 June 1999

References

1. Scalzo, A.A., N.A. Fitzgerald, C.R. Wallace, A.C. Gibbons, Y.C. Smart, R.C. Burton, and G.R. Shellam. 1992. The effect of *cmv-1* resistance gene, which is linked to the natural killer cell gene complex, is mediated by natural killer cells. *J. Immunol.* 149:581–589.
2. Orange, J.S., and Biron, C.A. 1996. An absolute and restricted requirement for IL-12 in natural killer cells IFN-γ production and antiviral defense. Studies of natural killer and T cell responses in contrasting viral infections. *J. Immunol.* 156:1138–1142.
3. Orange J.S., B.P. Wang, C. Terhorst, and C.A. Biron. 1995. Requirement for natural killer cell–produced interferon γ in defense against murine cytomegalovirus infection and enhancement of this defense pathway by interleukin 12 administration. *J. Exp. Med.* 182:1045–1056.
4. Biron, C.A. 1997. Activation and function of natural killer cell responses during viral infection. *Curr. Opin. Immunol.* 9:24–34.
5. Britt, W.J., and C.A. Alford. 1996. Cytomegalovirus. *In* Fields of Virology. B.N. Fields, D.M. Knipe, P.M. Howley, R.M. Chanock, J.L. Melnick, T.P. Monath, B. Roizman, and S.E. Straus, editors. Lippincott-Raven Publishers, Philadelphia. 2493–2523.
6. Campbell, A.E., J.S. Slater, V.J. Cavanaugh, and R.M. Sternberg. 1992. An early event in murine cytomegalovirus replication inhibits presentation of cellular antigens to cytotoxic T lymphocytes. *J. Virol.* 66:3011–3017.
7. del Val, M., H. Hengel, H. Hacker, U. Hertlamb, T. Ruppert, P. Lucin, and U.H. Koszinowski. 1992. Cytomegalovirus prevents antigen presentation by blocking the transport of peptide-loaded major histocompatibility complex class I molecules into the medial-Golgi compartment. *J. Exp. Med.* 176: 729–738.
8. Rawlinson, W.D., H.E. Farrell, and B.G. Barrell. 1996. Analysis of the complete DNA sequence of murine cytomegalovirus. *J. Virol.* 70:8833–8849.
9. Fahnestock, M.L., J.L. Johnson, R.M.R. Feldman, J.M. Neveu, W.S. Lane, and P.J. Bjorkman. 1995. The MHC class I homolog encoded by human cytomegalovirus binds endogenous peptides. *Immunity*. 3:583–590.
10. Chapman, T.L., and P.J. Bjorkman. 1998. Characterization of a murine cytomegalovirus class I major histocompatibility complex (MHC) homolog: comparison to MHC molecules and to the human cytomegalovirus MHC homolog. *J. Virol.* 72:460–466.
11. Farrell, H.E., H. Vally, D.M. Lynch, P. Fleming, G.R. Shellam, A.A. Scalzo, and N.J. Davis-Poynter. 1997. Inhibition of natural killer cells by cytomegalovirus MHC class I homologue *in vivo*. *Nature.* 386:510–514.
12. Reyburn, H.T., O. Mandelboim, M. Valez-Gomez, D.M. Davis, L. Pazmany, and J.L. Strominger. 1997. The class I MHC homologue of human cytomegalovirus inhibits attack by natural killer cells. *Nature*. 386:514–519.
13. Braud, V.M., D.S.J. Allan, C.A. O'Callaghan, K. Soderstrom, A. D'Andrea, G.S. Ogg, S. Lazetic, N.T. Young, J.I. Bell, J.H. Phillips, et al. 1998. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Science.* 391:795–799.
14. Borrego, F., M. Ulbrecht, E.H. Weiss, J.E. Coligan, and A.G. Brooks. 1998. Recognition of human histocompatibility leukocyte antigen (HLA)-E complexes with HLA class I signal sequence–derived peptides by CD94/NKG2 confers protection from natural killer–mediated lysis. *J. Exp. Med.* 187:813–818.
15. Rajagopalan, S., and E.O. Long. 1999. A human histocompatibility leukocyte antigen (HLA)-G–specific receptor expressed on all natural killer cells. *J. Exp. Med.* 189:1093–1100.
16. Chang, C.S., L. Brossay, M. Kronenberg, and K.P. Kane. 1999. The murine nonclassical class I major histocompatibility complex–like CD1.1 molecule protects target cells from lymphokine-activated killer cell cytolysis. *J. Exp. Med.* 189: 483–491.
17. Kubota, A., S. Kubota, H.E. Farrell, N. Davis-Poynter, and F. Takei. 1999. Inhibition of NK cells by murine CMV-encoded class I MHC homologue m144. *Cell. Immunol.* 191: 145–151.
18. Kagi, D., B. Ledermann, K. Burki, P. Seiler, B. Odermatt, K.J. Olsen, E.R. Podack, R.M. Zinkernagel, and H. Hengartner. 1994. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature.* 369:31–37.
19. Smyth, M.J., V.R. Sutton, M.H. Kershaw, and J.A. Trapani. 1996. Xenospecific cytotoxic T lymphocytes use perforin- and Fas-mediated lytic pathways. *Transplantation.* 62:1529–1532.
20. Smyth, M.J., E. Krasovskis, and R.W. Johnstone. 1998. Fas ligand-mediated lysis of self bystander targets by human papillomavirus-specific $CD8^+$ cytotoxic T lymphocytes. *J. Virol.* 72:5948–5954.
21. Smyth, M.J., J.M. Kelly, A.G. Baxter, H. Korner, and J.D. Sedgwick. 1998. An essential role for tumor necrosis factor in natural killer cell–mediated tumor rejection in the peritoneum. *J. Exp. Med.* 188:1611–1619.
22. van den Broek, M.F., D. Kagi, R.M. Zinkernagel, and H. Hengartner. 1995. Perforin dependence of natural killer cell-mediated tumor control *in vivo*. *Eur. J. Immunol.* 25:3514–3516.
23. Ljunggren, H.G., and K. Karre. 1985. Host resistance directed selectively against H-2–deficient lymphoma variants. Analysis of the mechanism. *J. Exp. Med.* 162:1745–1759.
24. Salazar-Mather, T.P., J.S. Orange, and C.A. Biron. 1998. Early murine cytomegalovirus (MCMV) infection induces liver natural killer (NK) cell inflammation and protection through macrophage inflammatory protein 1α (MIP-1α)–dependent pathways. *J. Exp. Med.* 187:1–14.
25. Leong, C.C., T.L. Chapman, P.J. Bjorkman, D. Formankova, E.S. Mocarski, J.H. Phillips, and L.L. Lanier. 1998. Modulation of natural killer cell cytotoxicity in human cytomegalovirus infection: the role of endogenous class I major histocompatibility complex and a viral class I homolog. *J. Exp. Med.* 187:1681–1687.
26. Cosman, D., N. Fanger, L. Borges, M. Kubin, W. Chin, L.

Peterson, and M. Hsu. 1997. A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules. *Immunity*. 7:273–282.
27. Grage-Griebenow, E., D. Lorenzen, R. Fetting, H.D. Flad, and M. Ernst. 1993. Phenotypical and functional characterization of Fc γ receptor I (CD64)-negative monocytes, a minor human monocyte subpopulation with high accessory and antiviral activity. *Eur. J. Immunol.* 23:3126–3135.
28. Hosken, N.A., and M. Bevan. 1992. An endogenous antigenic peptide bypasses the class I antigen presentation defect in RMA-S. *J. Exp. Med.* 175:719–729.
29. Kurepa, Z., C.A. Hasemann, and J. Forman. 1998. Qa-1^b binds conserved class I leader peptides derived from several mammalian species. *J. Exp. Med.* 188:973–978.
30. Salcedo, M., P. Bousso, H.G. Ljunggren, P. Kourilsky, and J.P. Abastado. 1998. The Qa-1^b molecule binds to a large subpopulation of murine NK cells. *Eur. J. Immunol.* 28:4356–4361.
31. Tay, C.H., and R.M. Welsh. 1997. Distinct organ-dependent mechanisms for the control of murine cytomegalovirus infection by natural killer cells. *J. Virol.* 71:267–275.