

Ly49P recognition of cytomegalovirus-infected cells expressing H2-D^k and CMV-encoded m04 correlates with the NK cell antiviral response

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Natural killer (NK) cells are crucial in resistance to certain viral infections, but the mechanisms used to recognize infected cells remain largely unknown. Here, we show that the activating Ly49P receptor recognizes cells infected with mouse cytomegalovirus (MCMV) by a process that requires the presence of H2-D^k and the MCMV m04 protein. Using H2 chimeras between H2-D^b and -D^k, we demonstrate that the H2-D^k peptide-binding platform is required for Ly49P recognition. We identified m04 as a viral component necessary for recognition using a panel of MCMV-deletion mutant viruses and complementation of m04-deletion mutant ($\Delta m04$) virus infection. MA/My mice, which express Ly49P and H2-D^k, are resistant to MCMV; however, infection with $\Delta m04$ MCMV abrogates resistance. Depletion of NK cells in MA/My mice abrogates their resistance to wild-type MCMV infection, but does not significantly affect viral titers in mice infected with $\Delta m04$ virus, implicating NK cells in host protection through m04-dependent recognition. These findings reveal a novel mechanism of major histocompatibility complex class I-restricted recognition of virally infected cells by an activating NK cell receptor.

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Humans (1) and mice (2) lacking NK cells are vulnerable to certain viral infections. NK cells patrol for aberrant cells, which they eliminate through cytotoxic activity and secretion of cytokines (3). NK cell activation depends on a complex array of inhibitory and activating receptors. Inhibitory receptors recognizing MHC class I molecules, including human killer cell immunoglobulin-like receptors (KIR) and mouse Ly49 receptors, play a predominant role. Both receptor families contain activating receptors, but the ligands are not well characterized. Viruses have devised numerous strategies to modulate the function of NK cells and have influenced the evolution of NK cell receptors.

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The identification of mouse genes that protect from mouse cytomegalovirus (MCMV) infection has provided insights into the NK cell-mediated control of viral proliferation. In C57BL/6 mice, NK cells expressing the activating Ly49H receptor that binds directly to m157, which is a MCMV-encoded cell surface glycoprotein (3), control viral replication early after infection (4). The essential role of Ly49H was confirmed by the transfer of MCMV resistance to genetically susceptible mice by *Ly49h* transgenesis (5), whereas absence of *Ly49h* in C57BL/6 mice abolishes resistance (6). Moreover, C57BL/6 mice are susceptible to MCMV

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mutants lacking *m157* ($\Delta m157$) (7). Collectively, these data demonstrated the importance of activating NK cell receptor-specific recognition of a virus-encoded ligand in host resistance.

Despite lacking *Ly49h*, MA/My mice are resistant to MCMV (8, 9), which depends on the specific combination of MA/My alleles at *Ly49* and MHC loci. The activating receptor Ly49P in MA/My mice recognizes MCMV-infected cells, and Ly49P-dependent activation is abrogated by an anti-H2-D^k antibody (9). These results suggested that MA/My resistance is conferred by NK cell-mediated recognition of infected cells by a mechanism involving Ly49P, H2-D^k, and an additional molecule expressed during MCMV infection.

MCMVs possess numerous genes dedicated to manipulating the host immune response. Three MCMV gene products alter host MHC class I expression: *m04*, *m06*, and *m152* (10). *m152* retains peptide-loaded MHC class I in the endoplasmic reticulum cis-Golgi intermediate compartment (11). *m04* and *m06* carry cytoplasmic (CT) motifs involved in cargo-sorting pathways; *m06* redirects MHC class I to the late endosome-lysosome pathway for degradation, thus preventing antigen presentation (12). *m04* associates with MHC class I in the ER, and these complexes travel to the cell surface (13, 14); however, *m04* does not down-regulate MHC class I.

Here, we found that Ly49P recognition depends on the following: H2-D^k expression on the infected cell, the peptide-binding groove of H2-D^k, and *m04*. Infection of MA/My mice with a *m04*-deletion MCMV mutant resulted in increased susceptibility, suggesting that Ly49P-mediated NK cell recognition of H2-D^k complexed with *m04* on the infected cell is necessary to attenuate virus replication.

RESULTS AND DISCUSSION

H2-D^k is necessary for Ly49P recognition of MCMV-infected cells

In view of MCMV's interference with host MHC class I expression, we examined whether sufficient amounts of H2 are expressed on the surface of infected cells to permit recognition by Ly49P (Fig. 1 A). BALB.K mouse embryonic fibroblasts (MEFs) were infected with MCMV expressing GFP (15), followed by staining with anti-H2-D^k or anti-H2-K^k antibodies (Fig. 1 A). Flow cytometric analysis revealed populations of cells that were noninfected (GFP-negative), mildly infected (GFP-dim), and highly infected (GFP-bright). We observed increased MHC class I expression on the GFP-negative cells, likely caused by up-regulation by type I IFN secreted by virus-infected cells. Importantly, GFP-dim MCMV-infected cells expressed H2-D^k and -K^k on their surface (Fig. 1 A). Therefore, MHC class I expression is diminished, but not totally ablated, in MCMV-infected cells (16).

Activation of Ly49P-expressing NFAT-GFP reporter cells co-cultured with MCMV-infected H2^k MEFs is abolished by anti-H2-D^k, but not anti-H2-K^k, blocking antibodies (9). We transduced NIH3T3 cells, which endogenously carry *H2^{q2}*, with H2-D^k and/or -K^k, and assessed surface

expression (Fig. 1 B). None of the transduced cells stimulated a human NK cell line transduced with Ly49P (NKL-Ly49P) to secrete INF- γ in the absence of infection (Fig. 1 C). However, when infected with MCMV, cells expressing H2-D^k stimulated NKL-Ly49P cells to produce INF- γ (Fig. 1 C). Ly49H-expressing NKL (NKL-Ly49H) cells secreted INF- γ when co-cultured with MCMV-infected cells, irrespective of H2 expression (Fig. 1 D). Therefore, H2-D^k, but not H2-K^k, is necessary for Ly49P activation in the presence of infection.

$\alpha 1\alpha 2$ domains of H2-D^k are necessary for Ly49P recognition

MHC class I molecules consist of a heavy chain with $\alpha 1$, $\alpha 2$, and $\alpha 3$ domains associated with β_2 -microglobulin (β_2m). The peptide-binding platform is composed of two parallel α helices positioned on top of a β -plated sheet, formed by the $\alpha 1$ and $\alpha 2$ domains. To determine which domains of H2-D^k are recognized by Ly49P, we generated chimeras by swapping domains between H2-D^k and H2-D^b (Fig. 2 A). $\alpha 1$ and $\alpha 2$ from H2-D^b were fused with the $\alpha 3$, transmembrane (TM), and CT domains of H2-D^k ($D_{\alpha 1\alpha 2}^b D_{\alpha 3}^k$). $\alpha 1$, $\alpha 2$, and $\alpha 3$ from H2-D^b were fused with the TM and CT domains of H2-D^k ($D_{\alpha 1\alpha 2\alpha 3}^b D_{\alpha 3}^k$). $\alpha 1$ and $\alpha 2$ from H2-D^k were fused with the $\alpha 3$, TM, and CT domains of H2-D^b ($D_{\alpha 1\alpha 2}^k D_{\alpha 3}^b$). $\alpha 1$, $\alpha 2$, and $\alpha 3$ from H2-D^k were fused with the TM and CT domains of H2-D^b ($D_{\alpha 1\alpha 2\alpha 3}^k D_{\alpha 3}^b$). Chimeric proteins were expressed in high amounts on the surface of NIH3T3 cells (Fig. 2 B).

These cells, which were infected or not infected with MCMV, were used to stimulate NKL-Ly49H or NKL-Ly49P cells. None of the uninfected cells induced significant amounts of INF- γ secretion. In contrast, MCMV-infected cells expressing H2-D^k, but not H2-D^b, activated the NKL-Ly49P cells (Fig. 2 C). When $\alpha 1$ and $\alpha 2$ of H2-D^k were replaced with those of H2-D^b, NKL-Ly49P cells were not stimulated (Fig. 2 C). Interestingly, when $\alpha 1$ and $\alpha 2$ of H2-D^b were replaced by those of -D^k, infected cells stimulated significantly higher amounts of INF- γ from NKL-Ly49P cells ($P < 0.01$; Fig. 2 C). Thus, the peptide-binding platform specified by $\alpha 1$ and $\alpha 2$ of H2-D^k is necessary for activating Ly49P recognition. The same region is important for contact between inhibitory Ly49 receptors and their MHC class I ligands (17).

m04 is critical for Ly49P recognition

MCMV genes involved in the manipulation of MHC class I might be involved in recognition by Ly49P. We tested MCMV deletion mutant viruses lacking members of the *m145* ($\Delta m144$ -158, $\Delta m151$ -158, $\Delta m151$ -165, $\Delta m152$, and $\Delta m157$) and the *m02* ($\Delta m1$ -22) gene families, including the *m04* and *m06* genes. Infected MEFs were used to stimulate Ly49P or Ly49H NFAT-GFP reporter cells. Absence of the *m145* family did not affect Ly49P recognition (Fig. 3). As expected, Ly49H reporters did not respond to MEFs infected with mutants lacking *m157* (Fig. 3) (9, 18). Although deletion of the *m1*-22 region

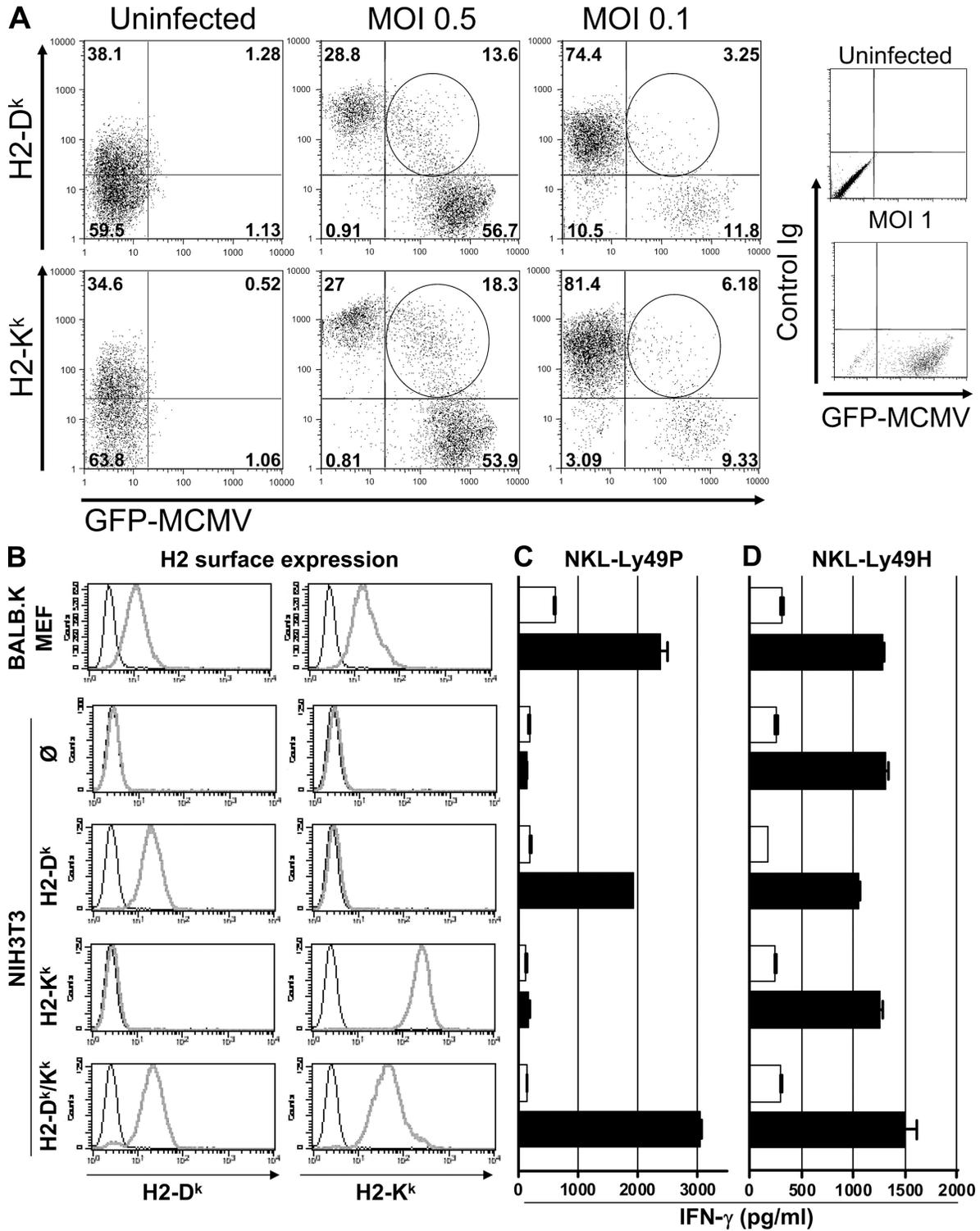


Figure 1. Activation via Ly49P requires H2-D^k. (A) H2^k proteins were detected on MCMV-infected cells. BALB.K MEFs were infected with GFP-MCMV (MOI 0.5 and 0.1) and stained with mAbs against: H2-D^k, H2-K^k, or an isotype-matched control antibody. GFP-DIM MCMV-infected cells expressing H2-D^k and H2-K^k molecules are shown in circles. (B) NIH3T3 cells were transduced with H2^k-encoding retroviruses and their surface expression was analyzed. BALB.K MEFs were a positive control. (C) NKL-Ly49P cells were co-cultured with uninfected (white bars) or MCMV-infected (black bars) cells. MCMV-infected or uninfected MEFs, NIH3T3 cells (∅), or NIH3T3 cells transduced with H2-D^k and/or -K^k were used as stimulators. IFN-γ was detected by ELISA. (D) NKL-Ly49H cells were a positive control. Data are representative of four independent experiments. Error bars represent the SD.

did not affect Ly49H recognition, it completely abolished Ly49P reporter cell activation. We also assayed deletion mutants lacking *m04* ($\Delta m04$), *m06* ($\Delta m06$), and/or *m152* ($\Delta m152$). Deletion of *m04*, but not *m06* or *m152*, completely abrogated Ly49P-mediated activation (Fig. 3).

m04 complements $\Delta m04$ MCMV in Ly49P-mediated activation

To determine whether m04 protein is sufficient to activate Ly49P reporter cells, we transiently transfected *m04* into immortalized MEF.K cells. When *m04*-transfected MEF.K cells were used to stimulate Ly49P-NFAT-GFP reporters, no significant activation was detected (Fig. 4 A). Ly49P-mediated recognition was rescued when *m04*-transfected cells were infected with $\Delta m04$ MCMV. *m90* and *m157* failed to complement $\Delta m04$ MCMV infection (Fig. 4 A). Thus, *m04* is necessary, but not sufficient, for Ly49P recognition.

To verify m04 expression, we transduced MEF.K with a V5-tagged *m04* and sorted V5-m04-expressing cells (V5-m04 MEF.K). As a control, we transduced MEF.K with a mono-

meric red fluorescent protein (mRFP-MEF.K). Uninfected cells did not activate the Ly49P-NFAT-GFP reporters (Fig. 4 B). In contrast to mRFP-MEF.K, $\Delta m04$ MCMV infection of V5-m04-MEF.K stimulated reporter cells at levels comparable to WT MCMV. Using FACS, we confirmed the presence of V5-m04 and H2-D^k on the cell surface, and distinguished infected cells by intracellular anti-m06 staining (Fig. 4 C). H2-D^k was expressed on the surface of ~20% of the cells infected with mutant or WT virus. To examine V5-m04 expression during infection, we gated cells based on H2-D^k and m06 expression (Dk⁻m06⁺, Dk⁺m06⁺, and Dk⁺m06⁻; Fig. 4 D). Consistent with reports of the association of m04 with H2 (19), we observed a clear correlation between V5-m04 and H2-D^k (Fig. 4 D). The largest frequency of V5-m04-expressing cells and the highest amounts of V5-m04 were seen on Dk⁺m06⁺ cells, demonstrating co-expression of V5-m04 and H2-D^k at the surface of infected cells. These results suggest that a factor in addition to m04 protein and H2-D^k in the infected cell is required for Ly49P-dependent recognition.

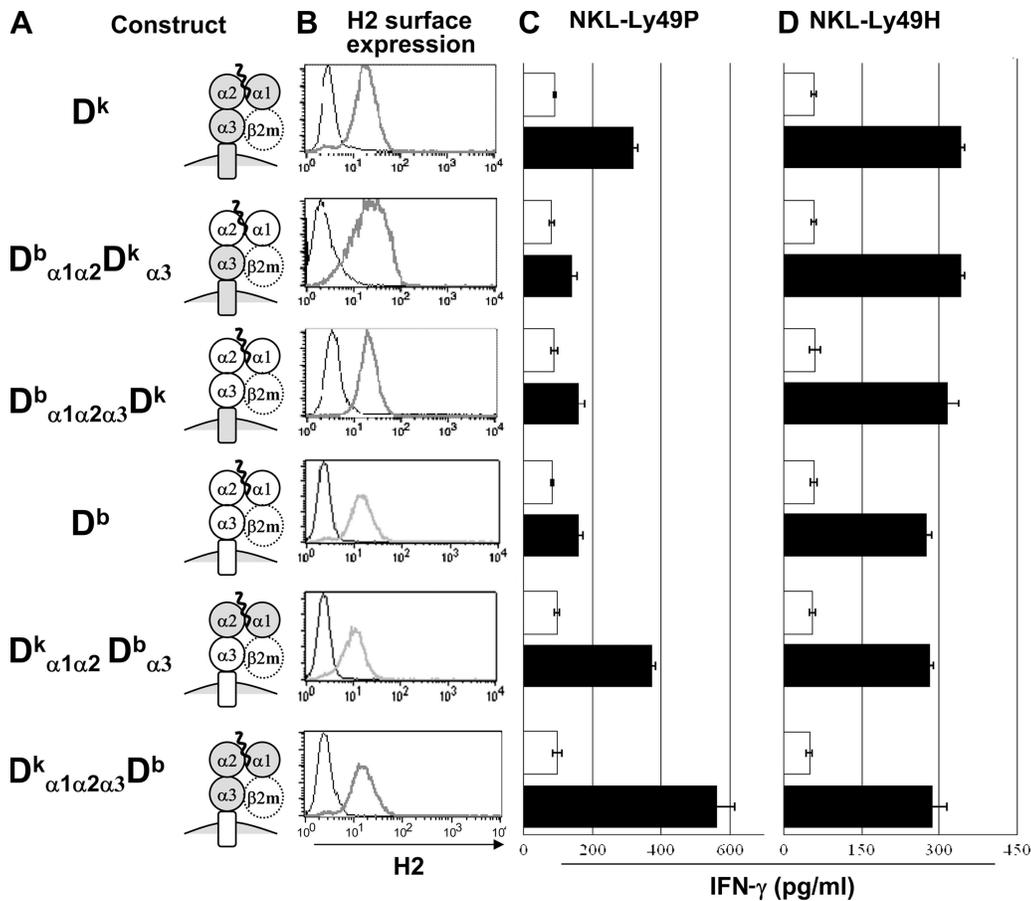


Figure 2. $\alpha 1\alpha 2$ of H2-D^k are necessary for Ly49P recognition. (A) Schematic representation. H2-D^k is represented in gray, H2-D^b in white, and $\beta 2m$ as white dotted circle. (B) Surface expression of the native H2 and chimeras on NIH3T3 cells was assayed using antibodies against the $\alpha 1\alpha 2$ domains of H2-D^k and/or -D^b. (C) NKL-Ly49P cells were co-cultured with uninfected (white bars) or MCMV-infected (black bars) cells. MCMV-infected or uninfected NIH3T3 cells expressing native H2 or chimeras were stimulators. Significant ($P < 0.01$) activation of NKL-Ly49P cells depended on the presence of $\alpha 1\alpha 2$ domains of H2-D^k on MCMV-infected cells. (D) NKL-Ly49H cells were a control. Data are representative of three independent experiments.

m04 is physically associated with H2 molecules. Peptide-loading facilitates m04–H2 complex formation, and β_2m is essential (19). Our results confirm that m04–H2 complexes are present on the cell surface and available for Ly49P recognition. However, m04 must play a role other than simply increasing the expression of MHC class I because Ly49P reporter cells were unresponsive to uninfected MEFs treated with type I IFN to increase H2 expression (9). m04 might cause an allosteric change in H2-D^k, thereby permitting interactions with Ly49P. However, expression of m04 in MCMV-infected fibroblasts does not affect stimulation of cytotoxic T cells, indicating native H2 folding and recognition (20). Alternatively, m04 might provide a specific peptide recognized by Ly49P in a H2-D^k-dependent manner. Although it remains to be determined whether m04 peptides are involved in Ly49P recognition, this might not be the case because MEF.K transfected with m04 failed to stimulate the Ly49P reporters in the absence of infection. m04 associates via its TM domain with H2 independently of MCMV infection. However, in uninfected cells, export of m04–H2 complexes to the cell surface is less efficient (Fig. 4 D) (19). Thus, other infection-induced proteins might be required for assembly or transport of H2-D^k–m04 complexes to the surface of infected cells for recognition by Ly49P.

m04 is critical for MCMV resistance in MA/My mice

Initially, m04 was proposed to inhibit NK cell activation, thereby favoring viral replication (13). Conversely, our results

suggest that m04 activates Ly49P-bearing NK cells, benefiting host defense. To evaluate the role of m04 *in vivo*, we infected MA/My mice with WT or $\Delta m04$ MCMV and measured viral load (Fig. 5 A). 4 d after infection (a.i.), mice infected with WT MCMV had a 100-fold lower viral titer in the spleen and liver than mice infected with $\Delta m04$ MCMV. Thus, removal of *m04* from MCMV genome diminishes MA/My resistance.

To determine the role of NK cells, MA/My mice were depleted of NK cells with anti-NK1.1 mAb. Splenic viral titers in untreated or NK cell-depleted MA/My mice infected with $\Delta m04$ MCMV were equivalent (Fig. 5 B). In contrast, untreated C57BL/6 mice infected with $\Delta m04$ MCMV showed a low viral load, equivalent to infection with WT MCMV, whereas NK cell-depleted C57BL/6 mice had a high viral burden when infected with either $\Delta m04$ or WT MCMV (Fig. 5 B). Although neutralizing antibodies against Ly49P are not available to formally prove that Ly49P-bearing NK cells are responsible for resistance, our studies strongly support a role for Ly49P–H2-D^k–m04 axis in the NK cell-mediated control of MCMV.

Concluding remarks

MCMV resistance in F₂ crosses between MCMV-resistant MA/My and MCMV-susceptible BALB/c mice indicated that only mice homozygous for *Ly49* and *H2^k* MA/My-derived alleles were resistant to MCMV (9). We identified *Ly49p* as the most likely gene involved in resistance based on:

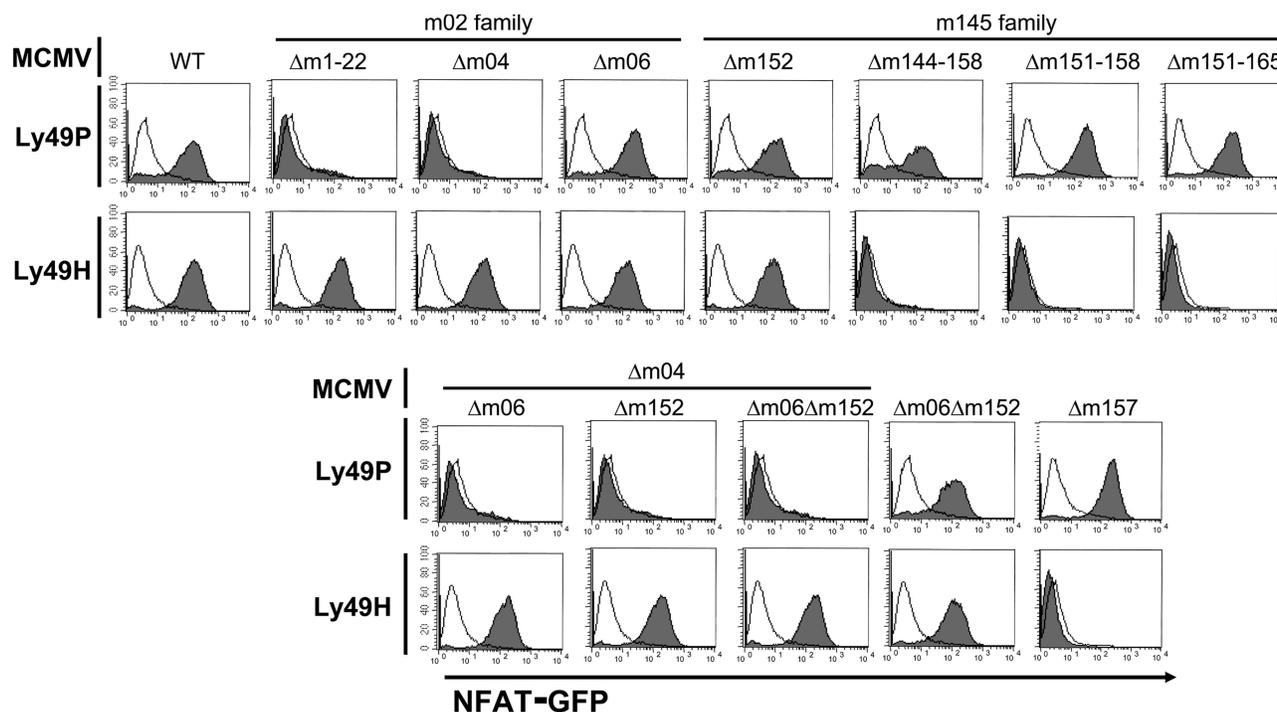


Figure 3. Ly49P-mediated recognition requires m04. Ly49P or Ly49H NFAT-GFP reporter cells were co-cultured with MEF.K infected with MCMV-deletion mutants and GFP expression was assessed (shaded histograms, infected; open histograms, uninfected). Data are representative of four independent experiments.

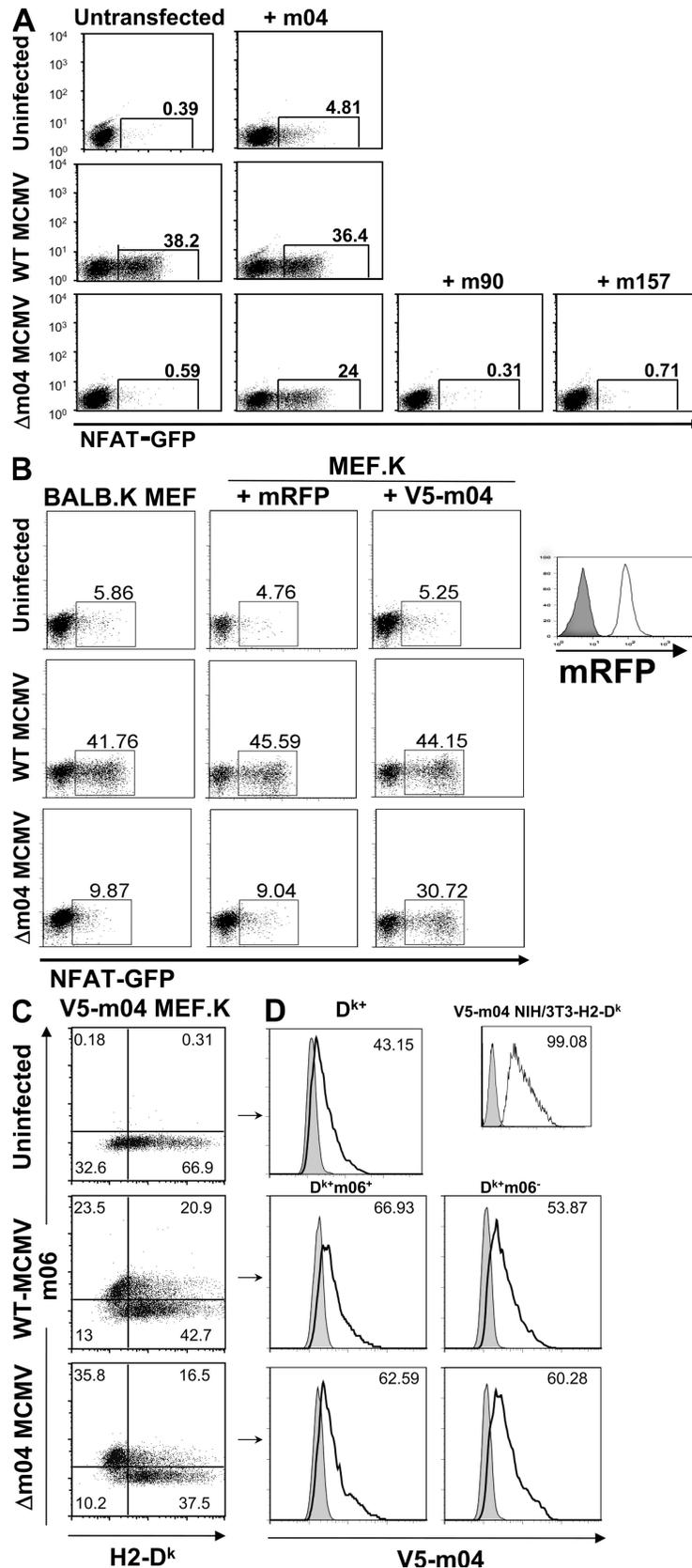


Figure 4. m04 complements Δ m04 MCMV infection in Ly49P reporter cell assays. (A) Transient m04 transfection complements the Δ m04 viral infection in the Ly49P reporter assays. Ly49P reporter cells were co-cultured with MEF.K either untransfected or transfected with m04 (+m04), m90

(a) its genetic location, (b) its absence in $H2^k$ MCMV-susceptible mice, and (c) its recognition of MCMV-infected cells using Ly49P reporter cell assays. Although the importance of the $H2^k$ haplotype for MCMV resistance was established previously, the precise mechanism had not been determined (9, 21). Here, we demonstrate that the peptide-binding $\alpha 1$ and $\alpha 2$ platform of H2-D^k is an absolute prerequisite for Ly49P recognition. Moreover, this recognition and NK cell-dependent resistance in MA/My mice is dependent on $m04$.

MCMV isolates from wild mice indicate that the $m04$ gene is very polymorphic, like $m157$ (22, 23). Variation in $m157$ is viewed as a result of the strong selective pressure applied by Ly49H⁺ NK cells, as demonstrated by a high rate of mutations in $m157$ isolated from MCMV passaged in $Ly49h$ -bearing mice (24, 25). Most $m04$ variation falls in the extracellular domain (26), which might influence the binding of Ly49P, suggesting that $m04$ polymorphisms emerge to escape NK cells.

Retention of a viral protein that enhances the killing of infected cells is counterintuitive, yet $m04$ might be beneficial for the virus. For example, through the stabilization of H2 on the surface of infected cells, $m04$ might render H2-D^k more accessible to inhibitory receptors and suppress NK cell activation in certain mouse strains. This has been proposed for $m157$, which is recognized not only by Ly49H but also by a closely related inhibitory receptor, Ly49I (18). Alternatively, $m04$ might confer host survival and thereby increase virus dissemination in the population, as proposed for $B15R$ in vaccinia virus, which encodes a soluble IL-1 receptor. Deletion of $B15R$ increases mortality in mice, suggesting that the viral blockade of IL-1 β diminishes the acute phase response and modulates severity of the disease (27). Regulation of antigen presentation by $m04$, in concert with $m06$ and $m152$, might influence the adaptive immune response to the virus' benefit (28). Regardless of how $m04$ evolved, the increased viral titers observed in MA/My mice infected with $\Delta m04$ MCMV demonstrate that $m04$ is crucial for effective NK cell-mediated control of viral replication. Given the fact that intact or NK cell-depleted MA/My mice infected with $\Delta m04$ MCMV had similar viral titers, it seems that recognition of $m04$ is responsible for most of the NK cell-mediated protective effect.

The Ly49P-H2-D^k- $m04$ model system provides new perspectives in understanding the relationship between KIR and HLA in human disease. Genetic epidemiological studies have identified several combinations of KIR and MHC loci associ-

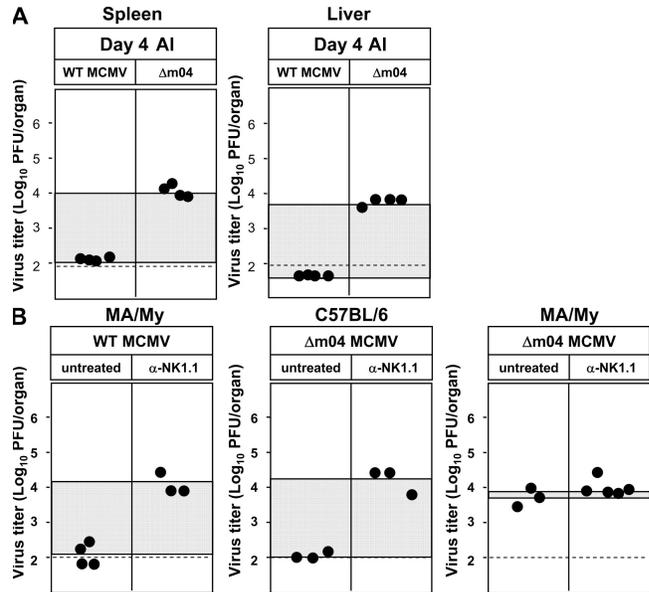


Figure 5. $m04$ deletion abrogates NK-cell mediated MA/My resistance. (A) MA/My mice were infected with 5×10^5 PFU of WT or $\Delta m04$ MCMV. 4 d a.i., viral loads in the spleen and liver were assessed. (B) MA/My and B6 mice were either untreated or depleted of NK cells 48 h before infection. 4 d a.i., viral titers in the spleen were assessed. Three to four mice per group were used. Data are representative of three independent experiments.

ated with resistance to HIV and hepatitis C virus (29); however, the molecular interactions responsible for these events remain unresolved. Our studies highlight the significance of activating NK cell receptor-MHC class I interactions in recognition of infection, warranting further investigation of their role in host resistance to other pathogens.

MATERIALS AND METHODS

Mice

Mice were purchased at The Jackson Laboratory. Animal protocols or experiments were approved by Canadian Council on Animal Care (CCAC) and McGill University Animal Resources Center.

Virus

$\Delta m04\Delta m06$, $\Delta m04\Delta m152$, $\Delta m06\Delta m152$, $\Delta m04\Delta m06\Delta m152$, $\Delta m144-158$, $\Delta m01-22$, $\Delta m151-m165$ (DMS94.5), $\Delta m151-m158$ (MC96.73), and WT BAC-derived (MW97.01) viruses were previously described (16, 30–33). 6–8-wk-old mice were infected i.p. with 5×10^3 PFU/mouse. Viruses were quantified by plaque assays (9). For NK cell depletion, mice were injected i.v. with 150 μ g anti-NK1.1 (PK136) mAb 48 h before infection.

(+ $m90$), or $m157$ (+ $m157$). Stimulators were uninfected or infected with WT or $\Delta m04$ MCMV. (B) Stable $m04$ expression complements the $\Delta m04$ viral infection in the Ly49P reporter assays. Ly49P reporter cells were co-cultured with mRFP (+mRFP) or V5- $m04$ (+V5- $m04$) positive MEF.K cells. Stimulators were treated as in A. The histogram shows mRFP expression in mRFP-MEF.K cells (black) and untransduced cells (gray). (C) $H2^k$ and V5- $m04$ are expressed on the surface of MCMV-infected cells. Uninfected and WT or $\Delta m04$ MCMV-infected V5- $m04$ MEF.K cells used in the reporter assay shown in (B) were costained with anti-H2-D^k, anti-V5, and anti- $m06$. Plots represent live-gated cells. (D) Top histograms display V5- $m04$ expression (black) in uninfected V5- $m04$ MEF.K (left) and V5- $m04$ NIH3T3-H2-D^k cells (right), compared with untransfected cells (gray). Middle and bottom histograms display WT or $\Delta m04$ MCMV-infected cells gated on the top right (D^k+ $m06$ ⁺) and bottom right (D^k+ $m06$ ⁻) quadrants of untransfected MEF.K (gray) and V5- $m04$ MEF.K (black) cells. The percentage of V5- $m04$ ⁺ cells in each gated population is indicated. Data are representative of three independent experiments.

Cells

Reporters. Ly49P- and Ly49H-transduced 2B4 NFAT-GFP reporter cells were previously described (9). NKL cells (gift from M. Robertson, Indiana University, Bloomington, IN) (34) were transduced with Ly49P^{MA/My} or Ly49H^{C57BL/6} in pMx-puro (gift from T. Kitamura, University of Tokyo, Tokyo, Japan) (18).

Stimulators. Primary BALB.K MEFs were prepared as previously described (9). BALB.K MEFs were immortalized (MEF.K) with polyoma large T antigen (gift from M. Fried, University of California, San Francisco, San Francisco, CA). H2-D^k, H2-D^b, or recombinant H2 cDNA in pMx-puro were used to transduce NIH3T3 cells. MEF.K were transiently transfected with pMx-puro containing *m04* (35), *m157*, or *m90*. *m04* lacking its endogenous leader sequence was cloned into pMx-neo that contains a human CD8 leader, followed by a V5 tag. mRFP cDNA was cloned into pMx-puro. MEF.K or NIH3T3-D^k cells were infected and sorted for high expression by flow cytometry.

Flow cytometry

MEF.K infected by GFP-MCMV (MOI 0.5 or 0.1) for 48 h were stained with biotin-conjugated anti-H2-D^k (15–5–5, ATCC) or anti-H2-K^k (16–3–22, ATCC), followed by APC-conjugated streptavidin (BD). NIH3T3 cells stably expressing H2-D^k, -K^k, -D^b, or chimeras were stained with FITC-conjugated anti-H2-D^k, -H2-K^k, or -H2-D^b (28–14–8; BD). Infected (MOI 0.5) or uninfected MEF.K, V5-m04 MEF.K, or V5-m04 NIH3T3-D^k cells were detached 24 h a.i., blocked with anti-CD16/CD32 mAb (eBioscience), and stained with anti-V5 (V5–10; Sigma-Aldrich), PE-conjugated donkey anti-mouse IgG (eBioscience), biotin-conjugated anti-H2-D^k, and APC-conjugated streptavidin. Cells were fixed, permeabilized, and stained with Alexa Fluor 488-conjugated anti-m06 antibody (CROMA 229) (36). Cells were analyzed on a FACSCalibur (BD).

Reporter assay

Stimulator cells were infected (MOI 0.5 or 1) with MCMV or MCMV-deletion mutants for 22 h. NFAT-GFP reporter cells were co-cultured overnight with stimulators in 48-well plates at a 1:1 ratio and analyzed by flow cytometry. NKL reporter cells were co-cultured overnight with stimulators at 5:1 ratio in 96-well plates, and IFN- γ was measured by ELISA (eBioscience).

Statistics

Differences between groups were calculated with two-way ANOVA analysis assuming not-repeated measures, followed by Bonferroni after tests. Results with $P < 0.05$ were considered significant.

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