Published in final edited form as:
Nat Genet. 2005 June ; 37(6): 593–599.

Epistasis between mouse *Klra* and major histocompatibility complex class I loci is associated with a new mechanism of natural killer cell–mediated innate resistance to cytomegalovirus infection

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Abstract

Experimental infection with mouse cytomegalovirus (MCMV) has been used to elucidate the intricate host-pathogen mechanisms that determine innate resistance to infection. Linkage analyses in F_2 progeny from MCMV-resistant MA/My (*H2*^{k}) and MCMV-susceptible BALB/c (*H2*^{d}) and BALB.K (*H2*^{k}) mouse strains indicated that only the combination of alleles encoded by a gene in the *Klra* (also called *Ly49*) cluster on chromosome 6, and one in the major histocompatibility complex (*H2*) on chromosome 17, is associated with virus resistance. We found that natural killer cell–activating receptor Ly49P specifically recognized MCMV-infected cells, dependent on the presence of the *H2*^{k} haplotype. This binding was blocked using antibodies to H-2D^k but not antibodies to H-2K^k. These results are suggestive of a new natural killer cell mechanism implicated in MCMV resistance, which depends on the functional interaction of the Ly49P receptor and the major histocompatibility complex class I molecule H-2D^k on MCMV-infected cells.

Worldwide, seven of ten individuals are infected with human cyto-megalovirus[1], which causes severe and fatal disease in neonates and immunocompromised individuals[2–4]. The crucial role of natural killer (NK) cells in the control of this infection has been extensively documented[5,6]. NK cells are important in the innate response to a variety of viral infections through lysis of infected target cells and production of an array of cytokines[6]. MCMV infections share many

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Note: Supplementary information is available on the Nature Genetics website.

COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

pathophysiological aspects with the human disease, providing an excellent model for elucidating the role of NK cells in the host response to viral infection. In addition, different inbred strains of mice vary markedly in susceptibility to MCMV, enabling a genetic approach to the mapping of susceptibility traits and to the identification of key genes governing NK cell function.

H2 genes in the major histocompatibility complex (MHC) and non-MHC genes determine resistance or susceptibility to MCMV in inbred strains of mice[7,8]. The *H2* complex influences susceptibility to the lethal effect of MCMV[7]. Strains such as C3H and CBA carry the protective $H2^k$ haplotype, whereas mice with the $H2^b$ (129) or $H2^d$ (BALB/c) haplotypes are more susceptible; susceptibility, in this case, is inherited as a dominant trait. At low-dose infection, in mouse strains of the C57BL/6 genetic background, *H2*-determined susceptibility is over-ridden by a dominant allele at the *Cmv1* locus, which acts early after infection at the level of host NK cells[9,10].

The *Cmv1* locus has two alleles in inbred mice: a dominant resistance allele, $Cmv1^r$, and a recessive susceptibility allele, $Cmv1^s$. Mouse strains possessing $Cmv1^r$ are five times more resistant to lethal MCMV infection and their viral titers in the spleen are 1,000–10,000 times lower than those of mouse strains possessing $Cmv1^s$ (ref. 9). *Cmv1* maps to distal mouse chromosome 6, in a region known as the NK cell gene complex (NKC)[11,12] because it encompasses clusters of genes and gene families of C-lectin-like–type receptors related to NK cell functions[13,14]. Among these are *Klrk1* (also called *Nkg2d*) and a group of more than 20 genes collectively known as *Klra* genes[13–15]. Recognition of classical MHC class I molecules by inhibitory Ly49 receptors is thought to provide a dominant inhibitory signal to NK cells against self in normal conditions[16].

$Cmv1^r$ encodes the activating receptor Ly49H[17–20], which recognizes MCMV-infected cells by a direct interaction with the m157 MCMV gene product[17,18]. Haplotype studies using genetic markers in the vicinity of *Cmv1* established that although the $Cmv1^r$ haplotype is unique to C57BL/6 strains, there are two independent origins for MCMV susceptibility in mice. On the basis of haplotype relatedness, MCMV-susceptible strains of mice were clustered into two groups, with mice similar to strain 129P3 (hereafter called 129) forming one subset and mice similar to strain BALB/c forming a second, unrelated subset[19].

The MCMV-resistant strain MA/My[12] lacks *Klra8* (also called *Ly49h*) mRNA and protein (M.P. Desrosiers, S. Girard Adam, A. Kiel-czewska and S.M. Vidal, unpublished data). In addition, depletion of MA/My NK cells with monoclonal antibody to NK1.1 abolished this resistance[12], suggesting the presence of another type of $Cmv1^r$ allele in this mouse strain. Here we show by haplotype analysis that the resistant strain MA/My is genetically divergent from the resistant strain C57BL/6 in the region of *Cmv1* and highly related to the MCMV-susceptible strains FVB/N (hereafter called FVB) and 129. Linkage analysis of the resistance phenotype indicated that MA/My mice possess a key determinant of resistance to MCMV, which maps to the NKC. Genetic and molecular analysis of this phenotype uncovered a new mechanism of host resistance against MCMV infection that involves interaction between the MA/My-encoded activating receptor Ly49P on NK cells and the MHC class I molecule $H\text{-}2D^k$ on virus-infected cells.

RESULTS

The MA/My NKC haplotype

To understand the molecular basis of resistance in the absence of Ly49H, we characterized the MA/My *Cmv1* region by haplotype analysis. We used 30 informative markers, either clustered in the *Klra* region or distributed in the NKC genomic domain and covering a physical distance

of 5 Mb (Fig. 1). Haplotype analysis (Fig. 1b) showed that MA/My is genetically distinct from C57BL/6 in the *Cmv1* region (16 of 30 markers in common). In particular, markers in the *Klra* region were polymorphic between these two strains, indicating the presence of a different *Klra* gene repertoire (7 of 10 different markers). Conversely, the MA/My haplotype was more similar to that of the MCMV-susceptible strains 129 and FVB, particularly in the *Klra* region (8 of 10 identical markers), indicating that this allele sharing might be the result of a common origin. Although the resistant MA/My strain and the susceptible FVB and 129 strains have a similar *Cmv1* haplotype, the difference in resistance to MCMV infection between the three strains could be explained, at least in part, by their *H2* haplotype, also implicated in MCMV infection. In fact, MA/My carries the protective $H2^{k}$ haplotype, whereas 129 and FVB have the susceptible $H2^{b}$ and $H2^{q}$ haplotypes, respectively.

Genetic analysis of MCMV resistance in MA/My

We investigated the mode of inheritance of the MA/My resistance trait in F_1 and F_2 progeny from crosses between MCMV-resistant MA/My and MCMV-susceptible BALB.K mice. Both strains share the same $H2^{k}$ haplotype. The read-out phenotype was spleen viral load 3 d after infection measured in $\log_{10}$ plaque-forming units (PFUs) recovered. (MA/My × BALB.K) F_1 mice had a mean viral titer (× 1 s.d.) of 3.35 ± 0.13 $\log_{10}$ PFUs, intermediate between the viral titers of MA/My (2.24 ± 0.62, $P < 2.8 \times 10^{-6}$) and BALB.K (4.43 ± 0.17, $P < 2.3 \times 10^{-6}$) mice (Fig. 2a), suggesting that resistance might be controlled by codominant alleles. In addition, the phenotypic distribution of the F_2 progeny was discontinuous, with three means at 2.5, 3.3 and 4.0 $\log_{10}$ PFUs (Fig. 2b), consistent with the presence of a major codominant gene effect.

Although MA/My mice do not express Ly49H, we did not exclude the possibility that another member of the *Klra* gene family or another NK cell receptor gene could contribute to MCMV resistance in MA/My. Therefore, we genotyped *Klra5* (also called *Ly49e*) and five additional markers spanning a total of 14 Mb in individual F_2 mice. All these markers were linked to the NKC and were common to both parental strains of mice (Fig. 1a and Table 1). On the basis of the empirical frequencies of the genotypic classes for *Klra5*, mice homozygous with respect to BALB.K alleles clustered toward the susceptible end of the distribution, and mice homozygous with respect to the MA/My alleles ($Klra5^{m}$) clustered toward the resistant end of the phenotypic spectrum (Fig. 2b). The logarithm of odds (lod) scores for the MCMV-resistance trait showed the strongest association with marker genes *Klrk1* and *Klra5* (Table 1), with a lod score of 22.7 ($P < 1.0 \times 10^{-6}$) under an additive mode of inheritance. The lod scores at the flanking markers *D6MIT135* (proximal) and *D6MIT291* (distal) were 20.8 and 18.7, respectively (Table 1). This linkage analysis located a new resistance locus that we named *Cmv3*. The one-lod support interval suggests that *Cmv3* is located in a region of ~8 Mb, indicating that *Klrk1* and *Klra* genes are good candidates for underlying the resistance trait. But analysis of MA/My and BALB/c *Klrk1* cDNA sequences indicated that they were identical and equivalently expressed in these mouse strains, excluding *Klrk1* as a potential resistance gene (A. Kielczewska and S.M. Vidal, unpublished observations).

H2 and *Klra5* interaction is associated with MCMV resistance

To investigate a possible role of *H2* in MA/My resistance, we studied the segregation of MCMV resistance in F_1 and F_2 crosses between MCMV-resistant MA/My ($H2^{k}$) and MCMV-susceptible BALB/c ($H2^{d}$) mice. Viral load in spleen 3 d after infection was used as the read-out phenotype. The mean value for the $\log_{10}$ of the PFUs recovered from the spleen of the (MA/My × BALB/c) F_1 population was 4.0 ±0.5 (Fig. 2a). This value was closer to that of the susceptible parental strain BALB/c (4.67 ± 0.19) than to that of the resistant parental strain MA/My (2.24 ±0.62; Fig. 2a), suggesting that MCMV resistance segregated as a recessive trait in this cross. Frequencies of viral titers in the spleen of 119 (MA/My × BALB/c) F_2 mice

showed a distribution that was suggestive of a more complex genetic control than in the previous cross, as shown by the clustering of the modes of *Klra5* genotype distributions (Fig. 2b).

To evaluate further the contribution of *H2* and NKC genes to MCMV resistance, we used the markers *IAA1* (located in *H2*) and *Klra5* (located in the NKC) as candidate genes to carry out an analysis of variance on viral spleen loads of 119 (MA/My × BALB/c) F_2 mice. The four additive-recessive combinations of *H2*-NKC models with and without an interaction term were fitted. All models that included *H2*, NKC and their interaction fitted consistently better. The most parsimonious model had a joint lod score of 9.3 and accounted for 30% of the phenotypic variation, consistent with the level of variation explained by *Cmv3* (Table 2). The *H2* recessive effect was almost twice as strong as the other two components combined. But less parsimonious models with other modes of inheritance were also highly significant. This might reflect a complex pattern of gene interactions with different effects on different *H2*-NKC haplotype combinations (Fig. 2c). The evidence for interaction was consistent across all the models (data not shown). The strength of the additive effect of *Klra5* (or other genes closely linked to it) was strongest in the presence of the $H2^{kk}$ background (Fig. 2c). Only mice homozygous with respect to $Klra5^{m}$ and $H2^{k}$ alleles were fully resistant to MCMV. Mice showed average $\log_{10}$ PFUs of 2.71 ± 0.35, in the range of MA/My controls (Fig. 2a,**c**), indicating that homozygosity with respect to the two loci was required for MCMV resistance.

Activating receptor Ly49P recognizes MCMV-infected cells

In accordance with haplotype analysis, we found that Ly49 receptors cloned from the MA/My strain were highly homologous to those of MCMV-susceptible strain 129 (ref. 20), suggesting that the two strains share a *Klra* gene repertoire. The *Klra9* (also called *Ly49i*) cDNA sequence from MA/My was identical to that of the 129 strain. The inhibitory receptor $Ly49I^{129}$ binds to the m157 viral protein[21], suggesting this as a possible susceptibility mechanism. Ten percent of MA/My NK cells stained with the m157-Ig fusion protein[21] (Table 3), excluding a hypothetical lack of Ly49I-m157 interaction as a means of MCMV resistance. The activating receptors, including Ly49P, Ly49R and Ly49U, shared 98–100% identity at the nucleotide level with their 129 counterparts (data not shown). These receptors were expressed on MA/My NK cells (**Supplementary** Fig. 1 online) and are good candidates for MCMV resistance in this strain through direct recognition of infected cells.

To test this hypothesis, we used a mouse T-cell hybridoma carrying the DAP12 adaptor signaling protein and a reporter gene *NFAT-GFP* and transfected it with cDNAs encoding Ly49P, Ly49R or Ly49U amplified from MA/My (Fig. 3). Growing the reporter cells on culture plates coated with monoclonal antibodies specific to the transfected receptors induced expression of green fluorescent protein (GFP), confirming that the reporters were functional (Fig. 3c). When Ly49 reporter cells were cultured with MCMV-infected mouse embryo fibroblasts (MEFs) from MA/My, only Ly49P reporters turned green, indicating the presence of a Ly49P ligand (Fig. 3a). This was not observed in the presence of uninfected MEF cells (Fig. 3a). Stimulation of *NFAT-GFP* was also absent when Ly49P reporter cells were cultured with Ba/F3 mouse pro-B cells expressing the MCMV-encoded ligand of $Ly49H^{C57BL/6}$ receptor, m157 (Fig. 3b), indicating that $Ly49P^{MA/My}$ recognized MCMV-infected cells by a mechanism that did not involve m157 detection.

Ly49P-MCMV specific activation depends on H-2D^{k}

To address the role of *H2* in Ly49P recognition of infected cells, we monitored stimulation of reporter cells transfected with Ly49P or with Ly49H (as a positive control) after culture with MCMV-infected MEF cells originating from mouse strains with different *H2* haplotypes (Fig. 4). Ly49P reporter cells did not respond when the infected target cells originated from BALB/

c ($H2^d$), FVB ($H2^q$) or C57BL/6 ($H2^b$) mice. In contrast, Ly49P receptors were activated in the presence of infected cells from MA/My ($H2^k$) or BALB.K ($H2^k$) mice (Fig. 4), indicative of a functional interaction between the H2 molecule and Ly49P receptor in the presence of MCMV infection.

Because H2 molecules are well-documented ligands of Ly49 receptors, we had to verify that Ly49P activation in reporter cells was MCMV-specific and not a result of an over-expression of MHC class I during infection. Whereas MEF cells that were cultured in the presence of recombinant interferon-β (IFN-β) had increased H2-D^k expression, as verified by surface staining (data not shown), they did not activate Ly49P reporters (Fig. 5a). Similarly, MEF cells infected with mouse herpesvirus 68 (MHV68) also did not activate Ly49P reporters, confirming the specificity of Ly49P for MCMV (Fig. 5a).

The previous results suggested that there is an $H2^k$ product (or products) recognized by Ly49P receptors. We determined that a monoclonal antibody to H-$2D^k$ (15-5-5S) efficiently blocked the activation of Ly49P reporter cells by BALB.K MCMV-infected cells ($H2^k$; Fig. 5b). In contrast, neither a monoclonal antibody recognizing H-$2K^k$ (36-7-5) nor an isotype-matched control antibody prevented Ly49P reporter activation. Furthermore, the presence of antibody against Ly49P, YE1/48, resulted in blocking of GFP production, showing that the reporter cell activation was Ly49P-specific. These results, together with the observation that Ly49R or Ly49U reporters were not activated (Fig. 3), support the role of Ly49P in mediating recognition of H-$2D^k$ on MCMV-infected cells.

DISCUSSION

In C57BL/6 mice, innate resistance to MCMV is mediated by the activating receptor Ly49H. We report here that MCMV resistance in MA/My is associated with *Cmv3*, a new NKC resistance locus linked to the *Klra* gene cluster. A fundamental difference between C57BL/6 and MA/My resistance alleles resides in their sensitivity to the *H2* background. Whereas the *Cmv1*^{r}-Ly49H phenotype is independent of *H2* environment, results from crosses between MA/My and BALB/c show that only a combination of homozygous *Cmv3*^{m} and $H2^k$ alleles is associated with MCMV resistance, indicative of different mechanisms of action. BALB/c has the $H2^d$ haplotype, and many of the inhibitory Ly49 receptors recognize $H2^d$ gene products as ligands. Therefore, it is possible that $H2^d$ contributes to MCMV susceptibility of BALB/c mice because many of their NK cells possess inhibitory Ly49 receptors, which bind to the products of these *H2* alleles. Heterozygous $H2^{kd}$ mice are also susceptible, suggesting that the $H2^d$ haplotype encodes additional ligands for inhibitory receptors in MA/My, resulting in increased NK cell inhibition[22]. These potential susceptibility mechanisms would, however, be overridden by the effect of *Cmv3*^{m}-$H2^k$ epistatic interaction.

In the investigation of the candidate genes for MCMV resistance, we provide evidence that *Klra16*$^{MA/My}$ (also called *Ly49p*$^{MA/My}$) is a resistance allele of *Cmv3*. *Klra16* maps to the confidence interval of *Cmv3* in the two crosses that we analyzed, totaling more than 690 meioses. Ly49P is associated with MCMV resistance in the $H2^k$ strain MA/My but not in the MCMV-susceptible FVB ($H2^q$) and 129 ($H2^b$) mice, even though the *Klra16* cDNAs are identical in these mouse strains (A. Makrigiannis, A. Kielczewska, S. Girard Adam, M.-P. Desrosiers and S.M. Vidal, unpublished observations). Moreover, Ly49P seems to be expressed on MA/My NK cells as far as we could establish using the YE1/48 monoclonal antibodies, which were previously used in the characterization of this receptor in 129 and NOD strains[23,24]. The exact number of Ly49P-positive NK cells cannot be determined owing to cross-reactivity of these antibodies with other Ly49 molecules in these mouse strains.

Finally, testing the candidate genes in a reporter cell assay provides evidence of Ly49P-specific recognition of MCMV-infected cells. From a panel of reporter cells expressing MA/My activating receptors, only the Ly49P reporters expressed GFP when cultured with MCMV-infected cells, indicating the presence of a ligand binding to Ly49P but not the other receptors. In addition, Ly49P recognition of the infected cells depended strictly on the *H2* background of the target cell because only $H2^k$ haplotype targets stimulated Ly49P, in concordance with the observed genetic interaction between the NKC and *H2* loci. Furthermore, we conclude from antibody blocking experiments that H-$2D^k$ in the presence of MCMV infection is a potential ligand for the Ly49P receptor. Together, these results support the idea that Ly49P receptor has a role in MCMV resistance through the NK cell-specific destruction of MCMV-infected cells.

Ly49P receptor activity was previously characterized in two other mouse strains. Using the rat NK cell line RNK-16 transfected with Ly49P from the NOD strain, it was shown that Ly49P induced lysis of susceptible targets[23], whereas immunoprecipitation assays showed that Ly49P from 129 precipitated with the adaptor protein DAP12 (ref. 24), confirming the role of Ly49P as an activating receptor. But the killing assay indicated that NOD Ly49P activity was dependent on H-$2D^d$–bearing targets[23], whereas the studies in the 129 strain showed that Ly49P interacted very weakly with H-$2D^k$ and H-$2D^d$ soluble tetramers. None of the previous studies tested the role of these MHC class I molecules in the context of MCMV infection. We present evidence that Ly49P target recognition is strictly dependent on the availability of H-$2D^k$ molecules on MCMV-infected cells. Depletion of MA/My NK cells with the YE1/48 monoclonal antibody before MCMV injection rendered mice susceptible to infection, underscoring the biological relevance of Ly49P in this model.

Activating and inhibitory isoforms of Ly49 MHC class I receptors are highly homologous in their extracellular domains. This probably results from the generation of the activating and inhibitory receptors by gene duplication and gene conversion. In cases where related activating and inhibitory receptors have been shown to bind to the same MHC class I ligand, the activating receptors generally seem to bind with much lower affinity to H2 than do the inhibitory receptors[25,26]. Our results suggest that Ly49P recognition of H-$2D^k$ in the infected cell is a new mechanism of activating NK cell receptor function, involving specific recognition of infected cells through classical MHC class I ligands. Although the exact nature of the interaction between Ly49P and H-$2D^k$ in MCMV-infected cells remains to be defined, it is plausible that ligation of the H-$2D^k$ molecule depends on the presence of a MCMV-specific peptide. Therefore, although the precise molecular machinery of target recognition is not yet known, our results indicate the possibility of peptide-specific recognition of MCMV by the Ly49P receptor. The requirement for peptide selectivity, previously well described for target-specific recognition by inhibitory MHC class I receptors[27,28], and *H2* allelic specificity renders the Ly49P–MCMV-infected cell encounter a highly specific mechanism of innate immunity mediated by NK cells, previously thought to be confined to cytotoxic T cells.

Peptide preference in MHC class I recognition by NK cell receptors is well documented for both Ly49 receptors and their functional homologs, human killer cell immunoglobulin receptors (KIR). In the case of the interaction between receptor KIR2DL2 and HLA-Cw3, direct contact between the KIR and the presented peptide occurs. There is less receptor contact to the side chain of the peptide than in T-cell receptor binding; therefore, the interaction is less sensitive to peptide content[29]. On the other hand, in the case of the Ly49C peptide-selective receptor, crystallographic studies show no contact between the receptor and the peptide. In fact, Ly49C interacts with the MHC class I molecule over a broad region beneath the peptide-binding platform. Dam and colleagues[30] proposed that, with Ly49C, certain peptides might exert interactions through the floor of the H-$2K^b$ binding grove, transmitted to the NK cell receptor by β_2M. This is a plausible mechanism for the Ly49P recognition of infected cells, resulting in the protection of the host against pathogens. Analogous to the mechanism of ligand

recognition by the promiscuous receptor Ly49C, Ly49P may also be able to recognize allosteric effects caused by a virus-encoded peptide. Alternatively, Ly49P may recognize an H2 molecule presenting a host-encoded peptide resulting from MCMV infection. Future studies will be necessary to determine whether recognition is specific for a single peptide or whether it results from a particular allosteric change in the H-2D^k molecule. Furthermore, if the peptide is of viral origin, the MCMV gene product implicated also awaits identification.

Although Ly49 and human KIR receptors are structurally unrelated, the two gene families share certain features, including polymorphism and variation in gene content and a similar pattern of clonal expression on different NK cells[31], as recently confirmed by transgenic models of KIR in mice[32]. Most notably, members of both receptor families recognize MHC class I antigens and use similar mechanisms for signal transduction. In humans, genetic epidemiological studies have identified several combinations of KIR and MHC loci associated with disease outcome, including those reported for human *KIR2DL3* and *HLA-C* protection against hepatitis C[33], and human *KIR3DS1* and *HLA-Bw4* activity delaying the progression of AIDS[34]. The evidence presented in this study emphasizes the importance of activating MHC class I NK cell receptors in recognition of infection, warranting further investigation of their role in host resistance to pathogens.

METHODS

Mice.

We purchased inbred mouse strains C57BL/6, MA/My, 129P3, FVB/N, BALB/c and BALB.K from the Jackson Laboratory. We produced F_1 and F_2 progeny from MA/My × BALB.K and MA/My × BALB/c crosses. Mice were kept at the animal facility of the University of Ottawa, in agreement with guidelines and regulations of the Canadian Council of Animal Care.

Virus stocks, mouse infections and virus titration.

We obtained the Smith strain of MCMV from the American Type Culture Collection. We produced virus stocks by salivary gland propagation in 3-week-old BALB/c mice as previously described[35]. To study susceptibility of mice to MCMV, we infected 6- to 8-week-old mice intraperitoneally with 5×10^3 PFUs MCMV. We determined viral titers on MA/My (n = 16), BALB.K (n = 25), BALB/c (n = 15), C57BL/6 (n = 22), 129 (n = 18), FVB (n = 20), (MA/My × BALB.K) F_1 (n = 6), (MA/My × BALB.K) F_1 (n = 10), (MA/My × BALB.K) F_2 (n = 226) and (MA/My × BALB/c) F_2 (n = 119) mice by plaque assay as described[35]. We collected spleens of mice 3 d after infection, homogenized them and centrifuged them at 500*g* for 20 min in a refrigerated centrifuge. We plated the supernatants for 1 h on monolayers of BALB/c MEF cells. After 3 d of incubation, we washed, fixed and stained the infected cells and counted MCMV plaques. Viral titers in the spleen are expressed as MCMV $\log_{10}$ PFUs. For antibody-depletion experiments, we injected mice intraperitoneally with 100–400 μg of antibody 2 d before infection.

Cell cultures.

We constructed 2B4 NFAT-GFP reporter cells and m157-transfected mouse Ba/F3 cells as described[17] and maintained them in complete RPMI-1640 medium (supplemented with 10% fetal bovine serum, penicillin, streptomycin and glutamine). We prepared MEF cells from strains MA/My, BALB/c, BALB.K, 129 and FVB as previously described[35], with modifications. We obtained embryos at 14–16 d of pregnancy, dissected them out of the embryonic sacs and removed livers and heads. We then finely minced the embryonic tissue with two forceps, washed it with phosphate-buffered saline and incubated it at 4 °C with trypsin overnight. The next, we dissociated the cells into a single-cell suspension and plated them on T175 flasks in complete DMEM medium (10% fetal bovine serum, 25 mM HEPES, penicillin,

streptomycin and glutamine). TpnT fibroblasts (an SV40-immortalized MEF cell line from C57BL/6 mice) were a gift from A. Hill (Oregon Health Sciences University, Portland, Oregon).

Genotyping.

We extracted genomic DNA from individual mouse tail tips using the alkaline method[36]. We carried out the haplotype analysis using 30 markers clustered in the *Klra* region or distributed in the NKC genomic domain. The DNA markers were microsatellites (*D6Mit52*, *D6Mit257*, *D6Mit135*, *D6Mit289*, D6Ott8, Ly49d, Klra8, Ly49g, Ly49a, D6Ott11, D6Ott32, 52A6L, D6Ott113, D6Ott117, D6Ott118, D6Ott115, 392D6L, 330B9L2, 242D11L, D6Mit13, *D6Mit290*, *D6Mit196*, *D6Mit374* and *D6Mit220*) or restriction-fragment length polymorphisms (*Klrk1*, *Klra5*, *282h8Sp6* and *200H7L*) or were detected by single-strand conformational polymorphism (*Ly49b*). The oligonucleotide primers and genotyping conditions were as described[11,19,37,38]. We analyzed NK1.1 expression by staining the NK cells with the PK136 monoclonal antibody (BD Pharmingen) using previously described conditions[12]. Together, the markers span a physical distance of 5 Mb (*D6MIT52*–*D6MIT220* interval). For the linkage analysis, we genotyped 226 (MA/My × BALB.K) F_2 mice and 119 (MA/My × BALB/c) F_2 using the *Klra5* marker representing the NKC region and *IAA1* markers representing the *H2* region. To discriminate between the *Klra5*^{c} (BALB/c, BALB.K) and *Klra5*^{m} (MA/My) alleles, we carried out amplification with the *Klra5* marker followed by digestion with *Hinc*II (New England Biolabs) as described[13]. To distinguish the *H2*^{d} (BALB/c) and *H2*^{k} (MA/My, BALB.K) haplotypes, we carried out amplification with *IAA1* primers followed by digestion with *Pst*I (New England Biolabs). PCR and enzyme digestion conditions and oligonucleotide sequences for the *IAA1* primers were previously reported[39]. For fine-mapping on chromosome 6, we genotyped (MA/My × BALB.K) F_2 mice using the *Klrk1* restriction-fragment length polymorphism marker and the microsatellite markers *D6MIT300*, *D6MIT52*, *D6MIT135* and *D6MIT291* (Mouse MapPairs; Research Genetics), spanning 14 Mb. Genotyping with *Klrk1* and microsatellite markers was done as previously described[11,37].

MA/My Klra cDNA repertoire.

We isolated total RNA from MA/My mice with TRIzol reagent (Life Technologies) and enriched it for poly $(A)^+$ using an Oligotex mRNA Midi kit (Qiagen). We used oligo(dT) primer and SuperScript II polymerase (Life Technologies) for reverse transcription. We amplified *Klra* transcripts with Advantage cDNA polymerase (Clonetech) by using *Klra* universal primers[40]. We purified the PCR product with a QIAEX II Gel Extraction Kit (Qiagen) and directly ligated it into pGEM T-Easy Vector System, in accordance with the manufacturer's instructions (Promega). We transformed ligation products using competent DH5α bacterial cells (InVitro-Gen) and grew them overnight at 37 °C on nylon membranes on Luria-Bertani broth agar plates. We duplicated the nylon membranes and treated them with 10% SDS buffer for 3 min to obtain optimal lysis, followed by a 5-min denaturing, neutralizing and washing step. We fixed DNA on membranes by ultraviolet cross-linking (70,000 μJ cm^{-2}). The duplicated set of membranes was independently hybridized with ITIM (inhibitory)- and non-ITIM (activating)-labeled (γ32-P-dATP) degenerated primers to discriminate inhibitory and activating *Klra* transcripts[40]. We exposed filters on Kodak X-ray films for 16 h and identified positive clones on the transformed plates. We dissolved single positive colonies in water and used them as DNA templates for PCR using *M13* universal primers to amplify the cDNA inserts. We purified plasmids from 20 positive clones with a Qiagen mini-kit and sequenced them by using a Licor automated system with SequiTherm EXCEL Kits (LC technology, Epicentre). We carried out DNA analysis of these clones using standard parameters of nucleotide-nucleotide BLAST (blastn) and protein-protein BLAST (blastp) programs. We used the ClustalW program for multiple sequence alignments.

Statistical analysis.

For the 226 (MA/My × BALB.K) F_2 mice, the genotypic data of markers on chromosome 6 were associated with their respective MCMV $\log_{10}$ PFU counts in the spleen using the MapManager QTb program41. This program, based on single-marker regression, allows identification of the loci that affect quantitative traits. For the 119 (MA/My × BALB/c) F_2 mice, the contribution of *Klra5* and *H2* alleles to the segregation of the phenotype was estimated under the linear model phenotype = *m* + NKC + H2 + NKC:H2 + *e*, where NKC and H2 represent factors that depend on the mode of inheritance proposed, *m* is the common mean value, NKC:H2 is an interaction term and *e* is the usual independent normally distributed random deviations. For the additive mode of inheritance, NKC and H2 represent the number of MA/My alleles at each locus. For the recessive mode of inheritance, NKC and H2 are the indicator variables of the homozygous BALB/c and MA/My background, respectively. The four possible additive-recessive combinations of H2-NKC models with and without an interaction term were fitted. We assessed the magnitude of the contribution for each term in the model by its *P* value, obtained by 1,000,000 bootstrapped samples, and partial η^2. Partial η^2 is computed as $\eta^2 = SS_{factor}/(SS_{factor} + SS_{error})$, where SS_{factor} is the type 3 associated sum of squares with the factor in the analysis of variance table, and SS_{error} is the sum of squares corresponding to the residual variation[42]. We carried out statistical and graphical analyses using R software.

Cell reporter assay.

We transduced NFAT-GFP 2B4 T-cell hybridoma reporter cells with cDNAs encoding MA/My Ly49P, Ly49R or Ly49U cloned into *Bam*HI and *Eco*RI sites of the pMx-puro vector as previously described[17]. We prepared primary MEF cultures from MA/My, BALB/c, FVB or BALB.K mice, as described above, infected with MCMV (Smith strain) at a multiplicity of infection of 1.0 for 22 h and used these as stimulator cells for the reporter assay. We cultured reporter cells overnight with the stimulator cells in 48-well plates in a 1:1 ratio. Activation of the NFAT-GFP reporter gene was detected by flow cytometric analysis and analyzed on a FACSCalibur flow cytometer with Cell Quest software (Becton Dickinson) and WinMDI (J. Trotter, Scripps Research Institute). In antibody blocking assays, reporter or stimulator cells were preincubated with a saturating concentration (>50 μg ml^{-1}) of an isotype-matched control immunoglobulin, 15-5-5S (anti-H-$2D^k$) or 16-3-22S (anti-H-$2K^k$) antibodies. We detected surface expression of H-$2K^k$ and H-$2D^k$ by using fluorescein isothiocyanate–conjugated 16-3-22S and 15-5-5S (anti-H-$2D^k$) antibodies (BD PharMingen) and measured fluorescence by flow cytometry.

URLs.

BLAST programs are available at http://www.ncbi.nlm.nih.gov/BLAST/. ClustalW is available at http://www.ebi.ac.uk/clustalw/. R software is available at http://www.R-project.org/.

GenBank accession numbers.

Klra18$^{MA/My}$ mRNA, AY971805; *Klra21*$^{MA/My}$ mRNA, AY971806; *Klra16*$^{MA/My}$ mRNA, AY971807.

Acknowledgements

We thank S.-H. Lee for the initial characterization of the MA/My strain; F. Takei for providing the YE1/48 antibody for depletion experiments; D. Albert and M.-H. Lacombe for technical support; M. Reuben for editorial assistance; and P. Gros, E. Schurr and M. Fujiwara for critical reading of the manuscript. This work was supported by grants from the Canadian Institutes of Health Research and the Canadian Genetic Diseases Network (Network of Centres of Excellence program). A.K., S.G.A. and J.-C.L.-O. were Canadian Institutes of Health Research Training Fellows in Infectious Diseases and Autoimmunity. A.K. was also supported by a McGill Majors Fellowship. M.B.L., T.P. and

L.L.L. are supported by a grant from the US National Institutes of Health. L.L.L. is an American Cancer Society Research Professor. S.M.V. is a Canada Research Chair.

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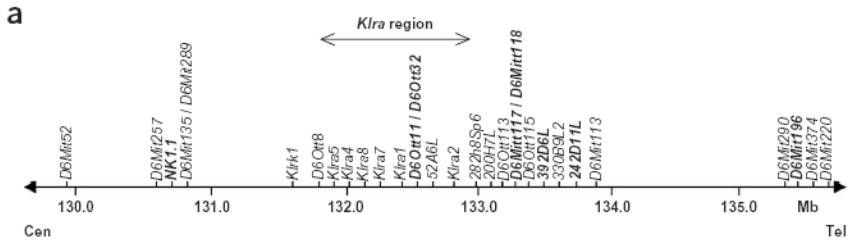

b

Klra region

	MCMV titer	D6Mit52	D6Mit257	NK1.1	D6Mit135	D6Mit289	Klrk1	D6Ott8	Klra5	Klra4	Klra8	Klra7	Klra1	D6Ott11	D6Ott32	52A6L	Klra2	282h8Sp6	200H7L	D6Ott113	D6Ott117	D6Ott118	D6Ott115	392D6L	330B9L2	242D11L	D6Mit113	D6Mit290
C57BL/6	2.06 ± 0.62	3	4	1	3	1	A	1	A	1	1	1	4	3	1	1	1	A	A	3	3	1	3	2	1	1	3	3
MA/My	2.24 ± 0.15	3	3	1	1	1	A	1	A	1	NP	3	2	2	NP	NP	NP	A	A	2	3	1	4	2	1	1	4	2
129/J	4.80 ± 0.05	2	1	NP	4	2	A	1	A	1	NP	3	2	4	NP	NP	1	A	A	2	3	1	3	2	1	1	3	2
FVB/N	5.02 ± 0.33	1	2	1	2	5	A	1	A	1	NP	3	2	2	NP	NP	3	A	A	2	3	1	2	2	1	1	5	3
BALB/c	4.67 ± 0.18	2	1	NP	6	3	B	1	B	NP	NP	2	1	1	NP	2	2	B	B	1	1	2	1	1	NP	NP	1	1

Figure 1.

Haplotype mapping on chromosome 6 in the vicinity of *Cmv1*. **(a)** Physical distance of markers used for haplotype mapping and genetic analysis. Markers in bold were arbitrarily positioned between well-defined markers. **(b)** Haplotype map of 30 polymorphic markers. Numbers indicate the relative size of PCR products (1 being the shortest) for microsatellite markers; letters indicate PCR–restriction-fragment length polymorphism markers. NP, no product.

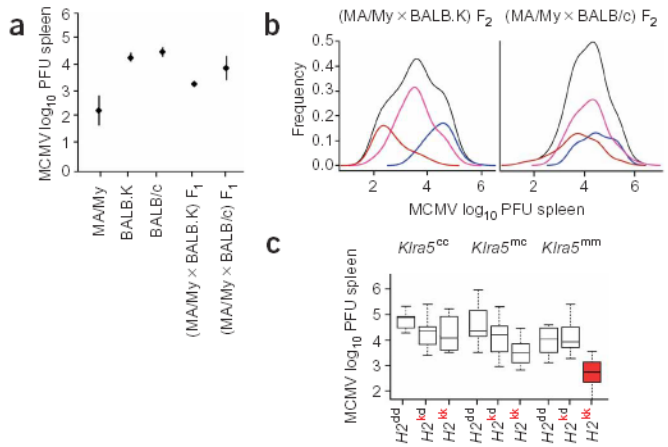

Figure 2.

Genetic analysis of MCMV resistance in MA/My mice. **(a)** MCMV viral titers in the spleen of MA/My (n = 16), BALB.K (n = 25), BALB/c (n = 15), (MA/My × BALB.K) F_1 (n = 6) and (MA/My × BALB/c) F_1 (n = 10) mice. Bars show standard deviation. **(b)** Empirical density (black line) of phenotypes of 226 (MA/My × BALB.K) F_2 mice (left) and 119 (MA/My × BALB/c) F_2 mice (right). Colored lines indicate the empirical density by genotypes at *Klra5*. In blue, distribution of homozygous BALB.K (n = 55) or BALB/c (n = 30) genotypes; in pink, distribution of the heterozygous genotype (MA/My × BALB.K, n = 118, or MA/My × BALB/c, n = 58); in brown, distribution of the homozygous MA/My genotypes (left, n = 53; right, n = 31) **(c)** Box plots of $\log_{10}$ PFU counts of *H2-Klra5* genotypes showing the combined effects of *H2* (MA/My, $H2^k$; BALB/c, $H2^d$) and NKC (MA/My, $Klra5^m$; BALB/c, $Klra5^c$) loci on spleen viral titers of 119 (MA/My × BALB/c) F_2 progeny. The median and interquartile range are shown. Whiskers extend to the most extreme value, which is less than 1.5 times the interquartile range. Solid dots denote outliers.

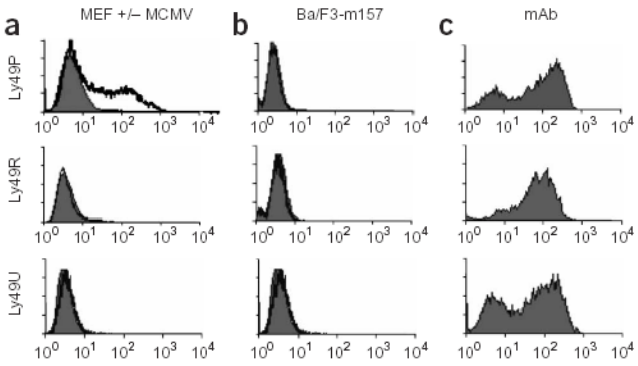

Figure 3.

Activation of Ly49P reporter cells by MCMV-infected cells. NFAT-GFP expression by reporter cells carrying individual Ly49 receptors was measured and GFP expression was analyzed by flow cytometry following culture of reporter cells: **(a)** with uninfected (filled histograms) or MCMV-infected (hollow histograms) MA/My MEF cells; **(b)** with Ba/F3 cells (filled histograms) or Ba/F3 cells expressing the MCMV protein, m157 (hollow histograms); **(c)** on plastic plates coated with monoclonal antibodies (mAb) to Ly49H-Ly49U, Ly49P and Ly49R.

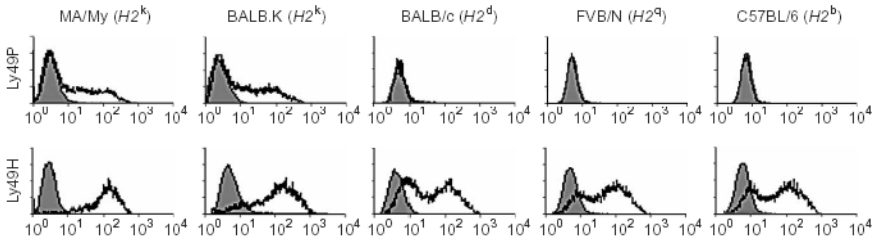

Figure 4.

Ly49P recognition of MCMV-infected cells is target MHC-dependent. NFAT-GFP stimulation by Ly49P and Ly49H reporter cells was determined after culture with MEF cells from MA/My (*H2*^{k}), BALB.K (*H2*^{k}), BALB/c (*H2*^{d}) and FVB (*H2*^{q}) inbred mouse strains and TpnT fibroblasts (*H2*^{b}) in the presence (hollow histograms) and absence (filled histograms) of MCMV infection. Ly49H reporter cells, which directly recognize the m157 MCMV protein even in the total absence of H2 molecules on the MCMV-infected cells[17], were equally activated when cultured with infected target cells from all of the above strains.

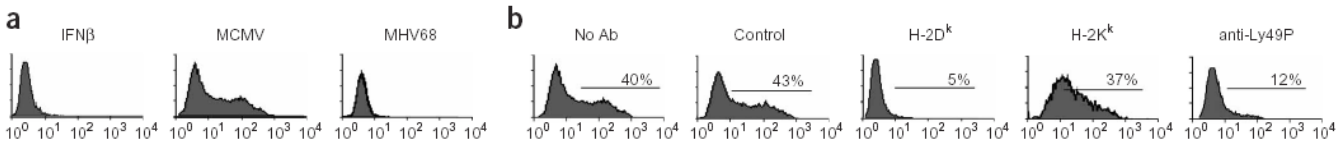

Figure 5.

Characterization of Ly49P interaction with an infected cell. **(a)** Ly49P activation is MCMV-specific. Ly49P reporter cell activation after culture with target MEF cells from BALB.K mice. Target BALB.K MEFs were either treated with IFNβ or infected with MCMV or mouse herpesvirus 68 (MHV68). **(b)** Ly49P recognition of MCMV-infected cells is blocked by antibodies to H-2D^k and Ly49P (YE1/48) but not by antibodies to H-2K^k. The antibody to Ly49P was used to block recognition of the infected cells by the Ly49P receptor to ensure its proper expression. NFAT-GFP stimulation of Ly49P reporter cells was measured after culture with MCMV-infected BALB.K MEF cells in the absence of blocking antibody or in the presence of isotype control antibody or antibodies to H-2D^k, H-2K^k or Ly49P.

Table 1

Linkage analysis in (MA/My × BALB.K) F_2 progeny

Locus	QTL analysis		
	Mb[a]	lod[b]	Variance (%)[c]
D6MIT300	123.6	18.6	32
D6MIT52	128.4	20.9	35
D6MIT135	129.5	20.8	35
Klrk1	130.3	22.7	37
Klra5	130.6	22.7	37
D6MIT291	137.5	18.7	32

The analysis was done on 226 (MA/My × BALB.K) F_2 mice.

[a] The position of each marker obtained from Ensembl.

[b] The lod ratio was used to measure the significance of each potential association of the trait with a locus.

[c] The amount of total trait variance explained by a quantitative trait locus (QTL) is expressed as a percentage.

Table 2

Analysis of variance of log10 PFUs in spleen in (MA/My × BALB/c) F_2 progeny

	Sum of squares	*F* value	*P* value	Partial η^2
Model	20.80	16.53	$<1.0 \times 10^{-6}$	0.301
NKC additive	1.66	3.95	4.9×10^{-2}	0.033
H2 recessive	12.11	28.87	$<1.0 \times 10^{-6}$	0.201
Interaction	4.60	10.97	1.2×10^{-3}	0.087

The analysis was done on 119 (MA/My × BALB/c) F_2 mice. *P* values are from 1,000,000 bootstrapped samples.

Table 3

Characteristics of mouse strains used in this study

Mouse strain	*H2* haplotype	MCMV	Ly49H	Ly49I isoform	Ly49P	m157-Ig–reactive NK cells*
C57BL/6	*H2*b	Resistant	+	Ly49IC57BL/6	−	60%
BALB/c	*H2*d	Susceptible	−	Ly49IBALB/c	−	0%
BALB.K	*H2*k	Susceptible	−	Ly49IBALB.K	−	0%
FVB/N	*H2*q	Susceptible	−	Ly49I129	+	10%
129P3	*H2*b	Susceptible	−	Ly49I129	+	10%
MA/My	*H2*k	Resistant	−	Ly49I129	+	10%

*The Ly49I^{129} isoform binds to the m157-Ig fusion protein. Although the presence of Ly49H receptor is sufficient to provide MCMV resistance, Ly49P must be in combination with *H2*^{k} haplotype.