

Ly49h is necessary for genetic resistance to murine cytomegalovirus

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Abstract Natural killer (NK) cells play critical roles in antiviral immunity. While the importance of effector mechanisms such as interferons has been demonstrated through knockout mice, specific mechanisms of how viruses are recognized and controlled by NK cells are less well defined. Previous genetic studies have mapped the resistance genes for murine cytomegalovirus (MCMV), herpes simplex virus-1 (HSV-1), and ectromelia virus to the NK gene complex on murine chromosome 6, a region containing the polymorphic *Ly49* and *Nkrp1* families. Genetic resistance to MCMV in C57BL/6 has been attributed to *Ly49H*, an activation receptor, through susceptibility of the recombinant inbred strain BXD-8 that lacks *Ly49h* (also known as *Klra8*) but derived about half of its genome from its DBA/2 progenitor. However, it

remained possible that epigenetic effects could account for the MCMV phenotype in BXD-8 mice. Herein, we report the generation of a novel congenic murine strain, B6.BXD8-*Klra8*^{Cmv1-del}/Wum, on the C57BL/6 genetic background to evaluate the effect of deletion of a single NK activation receptor, *Ly49H*. Deletion of *Ly49H* rendered mice much more susceptible to MCMV infection. This increase in susceptibility did not appear to be a result of a difference in NK cell expansion or interferon- γ (IFN- γ) production between the C57BL/6 and the B6.BXD8 strains. On the other hand, the deletion of *Ly49h* did not otherwise affect NK cell maturation or *Ly49D* expression and had no effect on susceptibility to HSV-1 or ectromelia virus. In conclusion, *Ly49h* is necessary for genetic resistance to MCMV, but not HSV-1 or ectromelia virus.

Keywords MCMV · *Ly49H* · Cytomegalovirus · *Cmv1* · Natural killer cells

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Introduction

Natural killer (NK) cells play important roles in innate immunity, particularly to viruses (French and Yokoyama 2003; Gazit et al. 2006). This is best illustrated by the clinical course of patients with natural killer cell defects which are characteristically manifested as recurrent infections with herpesviruses, such as cytomegalovirus (CMV) and herpes simplex virus-1 (HSV-1; Biron et al. 1989). Thus, greater understanding of how natural killer cells specifically recognize and control viral infections will have important clinical applications.

Given that CMV viruses are species-specific and that murine CMV (MCMV) shares significant biologic and genomic similarity with human CMV, MCMV has been

used extensively as an animal model for HCMV (Gonczol et al. 1985; Rawlinson et al. 1997). Several host factors, both major histocompatibility complex (MHC)-dependent and MHC-independent, account for MCMV resistance (Adam et al. 2006; Dighe et al. 2005; Grundy et al. 1981; Rodriguez et al. 2004; Scalzo et al. 1990). The first identified MHC-independent resistance gene, *Cmv1*, was mapped to distal mouse chromosome 6 in the vicinity of the NK gene complex (NKC) which encodes receptors expressed on NK cells (Scalzo et al. 1995). Identification of the Ly49H activation receptor as the product of the *Cmv1* locus was aided by the BXD-8 recombinant inbred (RI) strain (Brown et al. 2001b; Dokun et al. 2001; Lee et al. 2001).

Ly49H is a member of the Ly49 family of C-type lectin-like receptors, which are encoded in the NKC region on distal mouse chromosome 6. The Ly49 family is extremely polymorphic, displaying haplotypes with variations in gene number and allelic polymorphism for each gene (Proteau et al. 2004; Wilhelm et al. 2002). The significant difference in viral susceptibility between the resistant C57BL/6 and susceptible BALB/c has been attributed to the expression of Ly49H on NK cells in C57BL/6, whereas BALB/c mice lack the *Ly49h* gene (Brown et al. 2001a; Lee et al. 2001, 2003). Interestingly, loci for genetic resistance to two other viruses, HSV-1 and ectromelia virus, an orthopoxvirus, have also been mapped to the NKC (Brown et al. 2001a; Delano and Brownstein 1995). Although antibody-blocking experiments do not support a role for Ly49H in resistance to these viruses (data not shown), Ly49H has not been extensively studied in resistance by genetic approaches.

The BXD-8 RI strain arose from the consanguineous mating between C57BL/6 and DBA/2 progenitor strains for 20 generations. Consequently, half of the genome of this recombinant inbred strain is derived from the resistant C57BL/6 progenitor strain, while the other half from the susceptible DBA/2. BXD-8 mice carried C57BL/6 markers for distal chromosome 6 flanking the NKC region, but demonstrated increased susceptibility to MCMV compared to the C57BL/6 strain. This increased susceptibility has previously been shown to correlate with the absence of Ly49H expression by staining with the monoclonal antibody 3D10 on BXD-8 NK cells and a selective deletion in *Ly49h* by Southern analysis (Brown et al. 2001a). However, since approximately half of the BXD-8 genome is derived from the DBA/2 strain, it was not clear whether any DBA/2 alleles had an epistatic genetic effect on its increased susceptibility. Transgenic expression of a bacterial artificial chromosome containing the C57BL/6 allele of *Ly49h* into the susceptible BALB/c genetic background also conferred resistance to MCMV (Lee et al. 2003), but it remained possible that other background BALB/c alleles may modify the *Ly49h* effect.

To further analyze the role of *Ly49h* in NK cell function, particularly in infections, we backcrossed the BXD-8 RI strain onto C57BL/6 strain by marker-assisted backcrossing (speed congenics). We termed this novel strain B6.BXD8-*Klra8*^{*Cmv1-del*}/Wum, referred to by the shorthand of B6.BXD8 in this paper, and characterized the NK cell function of this strain.

Materials and methods

Animals C57BL/6NCR mice were purchased from National Cancer Institute. The RI strain BXD-8 was purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and was backcrossed to C57BL/6 for nine generations to generate the novel strain B6.BXD8-*Klra8*^{*Cmv1-del*}/Wum. At each successive generation, heterozygous pups carrying the deleted *Ly49h* allele were identified by flow cytometry. Microsatellite genotyping was performed by the Speed Congenics Core of the Rheumatic Diseases Core Center at Washington University to screen for C57BL/6 markers for the initial seven generations. Subsequently, two more generations were backcrossed onto C57BL/6 while following the heterozygous expression of Ly49H phenotype by flow cytometry. All animals were maintained in a specific pathogen-free facility at Washington University School of Medicine, and all experiments were approved by the Animal Studies Committee.

Southern analysis Genomic DNA for BXD-8 was purchased from Jackson Laboratory. Splenic genomic DNA for C57BL/6 and B6.BXD8 were extracted using Gene Pure following manufacturer's protocol. Digested DNA was separated on a 1% agarose gel and transferred onto a nitrocellulose membrane. Membrane was subsequently hybridized with the *Bgl*III-*Pst*I fragment of *Ly49H* complementary DNA (cDNA) labeled with ³²P-dCTP using Rediprime II System (Amersham, UK) following the manufacturer's protocol and image acquired using the Typhoon system (Amersham).

Plaque assays Viral titers were quantified using methods previously published (French et al. 2004). Briefly, whole spleens were harvested and frozen in 1 ml aliquots of D10 [Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum (Hyclone), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine]. Liver sections were weighed in 1 ml aliquots of D10 medium. Tissues were subsequently thawed, homogenized via dounce homogenizers, and used to infect NIH-3T12 fibroblast monolayers in triplicates in six-well plates. Titters were calculated as log(PFU/spleen) or log(PFU/100 mg of liver).

Flow cytometric analysis of natural killer cells Single-cell suspensions were prepared from spleens as previously described (French et al. 2004). Splenocytes were stained with APC-PK136 (NK1.1), PerCP-Cy5.5-2C11 (CD3), and PerCP-Cy5.5-1D3 (CD19) in supernatant produced by 2.4G2 hybridoma to block FcR2/3. The following antibodies were used to identify respective cell surface markers: PE-eBio12AB (Ly49A/D), PE-5E6 (Ly49C/I), FITC-4E4 (Ly49D), FITC-3D10 (Ly49H), PE-16a11 (NKG2AB6), and PE-CX5 (NKG2D). Except for the Ly49H antibody, which was made in-house, the other antibodies were purchased from BD Biosciences (San Jose, CA, USA). For intracellular IFN- γ staining, cells were permeabilized with Cytofix/Cytoperm (PharMingen, La Jolla, CA, USA) per manufacturer’s protocol and stained with PE-conjugated anti-IFN- γ (clone XMGI.2) purchased from BD Biosciences. To evaluate BrdU incorporation, each mouse was injected with 2 mg of BrdU intraperitoneally 3 h prior to spleen harvest. Splenocytes were stained with surface markers and then permeabilized using Cytofix/Cytoperm following the manufacturer’s protocol. Intracellular BrdU was determined by staining with FITC-anti-BrdU antibody (BD Biosciences) as previously described (Dokun et al. 2001).

Quantitative RT-PCR Total RNA was extracted from spleens using Trizol (Invitrogen, Carlsbad, CA, USA). cDNA was subsequently generated using oligo-dT primers while following the protocol for Superscript III First Strand Synthesis (Invitrogen). PCR primers for Ly49D were: forward 5’-TTCAGGGTTGCAGAACGAGATGAG-3’; reverse 5’-AGGATCCCGAGAGCTATCACAAATG-3’. The internal probe sequence was 5’-AAAGCTCGCCTCAGAGTTCCCTGGCA-3’. The primers for Ly49H were: forward 5’-CACAAGTCTTCAGGGTTGAACAGC-3’; reverse 5’-CAACAATTACCAGCCGAAGGGAAC-3’; internal probe 5’-TGGCCTAAGAGTCCCTTGGCAGCTCATTGT-3’. Messenger RNA (mRNA) expression was

normalized against β -actin expression and then compared to levels expressed in C57BL/6 splenocytes.

Results

Analysis of 110 microsatellite markers polymorphic between C57BL/6 and DBA/2 revealed that whereas the original recombinant inbred strain BXD-8 carried 52% of C57BL/6-derived genome, B6.BXD8 demonstrated 100% of C57BL/6-derived genome except for *Ly49h* (Fig. 1). Previous study from our laboratory demonstrated that BXD-8 carried a genomic deletion at the *Ly49h* locus in the midst of a chromosomal segment derived from C57BL/6 (Brown et al. 2001a, b). Southern analysis of B6.BXD8 genomic DNA digested with *Bgl*II and *Eco*RI, when hybridized with the 177-bp *Bgl*II-*Pst*I fragment of *Ly49h* cDNA, revealed the same patterns as those of BXD-8 genome (data not shown). The absence of a 3.5-kb band in B6.BXD8 in the *Bgl*II digest likely represented the genomic locus 82385903 to 82389340 in the C57BL/6 reference sequence (GeneBank accession number Mm6_39393_37). Furthermore, the absence of a 5-kb band in B6.BXD8 in the *Eco*RI digest likely represented the genomic locus 82385831 to 82390624 in C57BL/6. The exact junctions where this deletion occurred, however, was difficult to define given the high degree of sequence homology amongst Ly49 family members and the frequency of repeat sequences in this region. Repeated attempts to amplify genomic sequences in this region with PCR suggested that B6.BXD8 contained minimally a 20-kb genomic deletion spanning *Ly49h*, including the exon encoding the first start codon for *Ly49h* (data not shown). Consistent with this idea was the absence of detectable Ly49H mRNA by quantitative reverse transcription polymerase chain reaction (RT-PCR; Fig. 2a). Moreover, mRNA expression level of *Ly49d*, the gene immediately

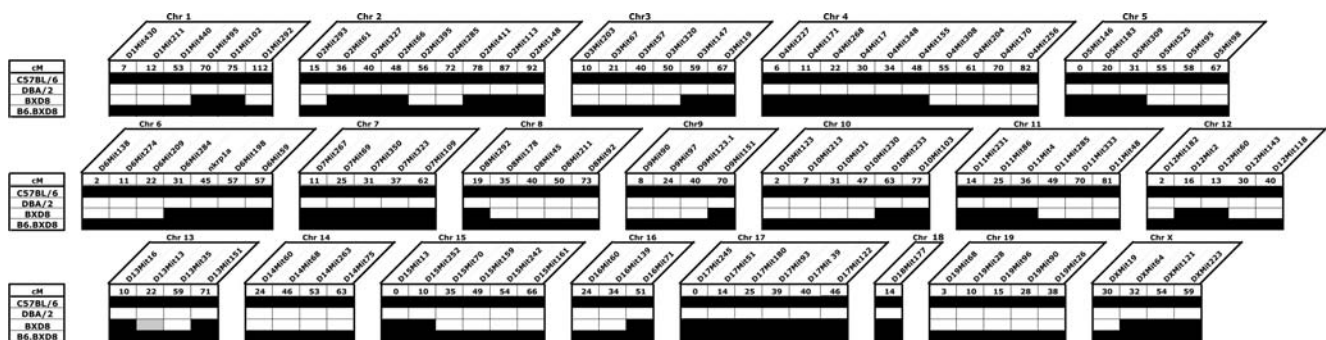


Fig. 1 Microsatellite genotyping analysis of B6.BXD8 strain. Numbers below microsatellite markers denote the distance of markers in centiMorgans according to the WI-Genetic Map (Hudson et al. 2001).

Black boxes denote C57BL/6 alleles, whereas white boxes indicate DBA/2 alleles. One shaded box (Chr 13) denotes a mixed-marker type

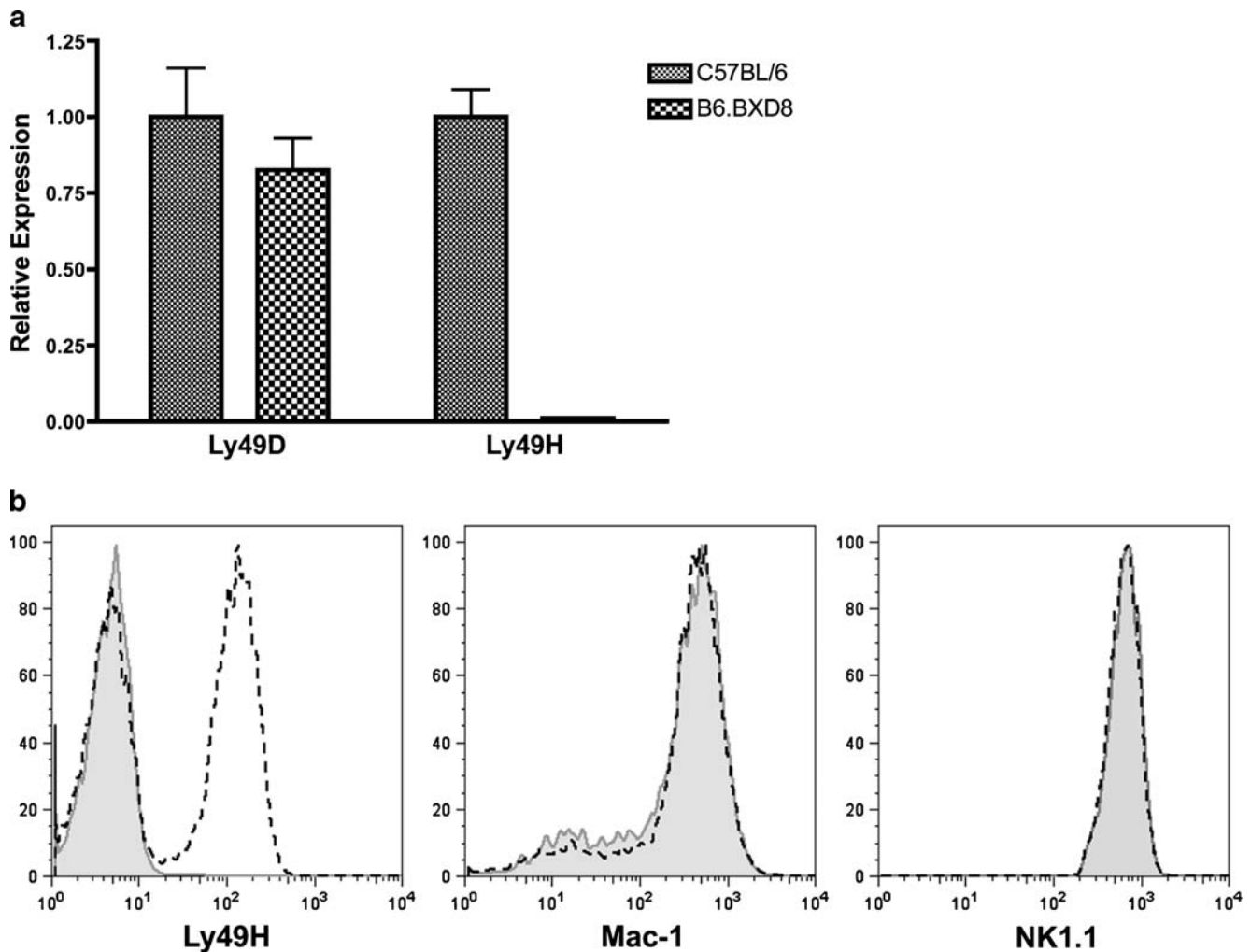


Fig. 2 Absence of *Ly49h* expression in B6.BXD8. **a** mRNA expression of Ly49H in C57BL/6 and B6.BXD8. As determined by quantitative RT-PCR, the relative expression represents ratios of Ly49D or Ly49H mRNA expression in B6.BXD8 splenocytes, normalized by β -actin, compared to those in C57BL/6 splenocytes. These results are representative of two independent experiments. **b** Flow cytometric analysis of C57BL/6 and B6.BXD8 NK cells. Fresh

splenocytes were labeled with a combination of anti-CD3, CD19, and NK1.1 antibodies in the presence of 2.4G2 hybridoma supernatant to block non-specific Fc γ RII/III binding. NK cell gate was defined as CD3⁺/CD19⁻/NK1.1⁺. The *dashed lines* represent NK cells from C57BL/6, while the *solid shaded curves* represent B6.BXD8. These results are representative of a minimum of two independent experiments

proximal to *Ly49h* in the NKC, was similar between C57BL/6 and B6.BXD8. Taken together, these data demonstrate that B6.BXD8 preserved the genomic *Ly49h* deletion from the original recombinant inbred strain BXD-8, while the rest of the genome is derived from C57BL/6.

At the protein level, flow cytometric analysis of NK cells from B6.BXD8 demonstrated no detectable surface staining with the anti-Ly49H-specific 3D10 monoclonal antibody as compared to 40% of NK cells from C57BL/6 (Fig. 2b, left panel). On the other hand, both C57BL/6 and B6.BXD8 NK cells expressed comparable levels of NK1.1, an NK cell-specific marker encoded by *Nkrp1c* in the neighboring NKC, adjacent to the *Ly49* locus. Furthermore, other receptors encoded in the NKC, such as *Nkrp1d* and *NKG2D*, were also expressed at similar levels in C57BL/

6 and B6.BXD8 (Fig. 3a). Finally, both B6.BXD8 and C57BL/6 NK cells expressed comparable levels of other Ly49 family members, Ly49A/D, Ly49D, Ly49C/I, and Ly49G (Fig. 3a), further supporting that B6.BXD8 specifically lacks Ly49H expression while demonstrating intact expression of known NKC surface receptors.

Further characterization of NK cells from B6.BXD8 mice demonstrated comparable levels of the maturation marker, Mac-1, as NK cells from C57BL/6 (Fig. 2b, middle panel). Inasmuch as Mac-1 is expressed on functionally mature NK cells, these data suggest full functional maturation of B6.BXD8 NK cells. Indeed, both B6.BXD8 and C57BL/6 NK cells produced similar levels of IFN- γ after NK1.1 activation (Fig. 3b). Thus, B6.BXD8 NK cells do not have any gross defect in IFN- γ production.

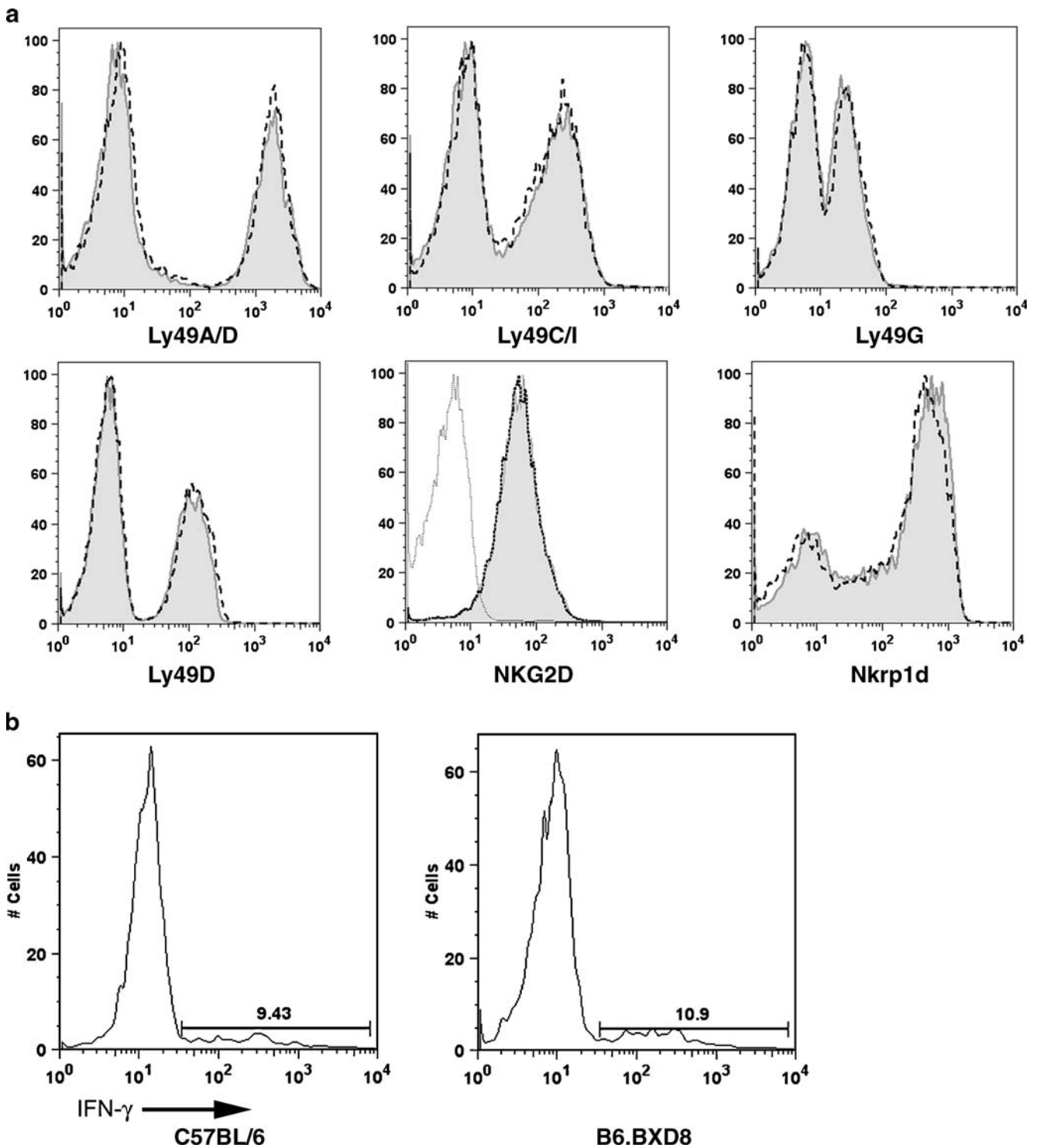


Fig. 3 Normal NKC receptor expression and function of NK cells in B6.BXD8. **a** Cell surface expression of NKC ligands on C57BL/6 and B6.BXD8 NK cells. The *dashed lines* represent NK cells from C57BL/6, while the *solid shaded curves* represent B6.BXD8. For the NKG2D staining, the *dotted unshaded curve* represents staining with the isotype control. Results were representative of two independent experiments. **b** NK cell activation after NK1.1 activation in vitro.

Freshly isolated splenocytes were plated in six-well plates coated with PK136 (anti-NK1.1 monoclonal antibody) at 5 $\mu\text{g}/\text{well}$ for 7 h in the presence of GolgiPlug (BD). Cells were subsequently permeabilized and intracellular IFN- γ expression analyzed following manufacturer's protocol (BD Biosciences). These results were representative of two independent experiments

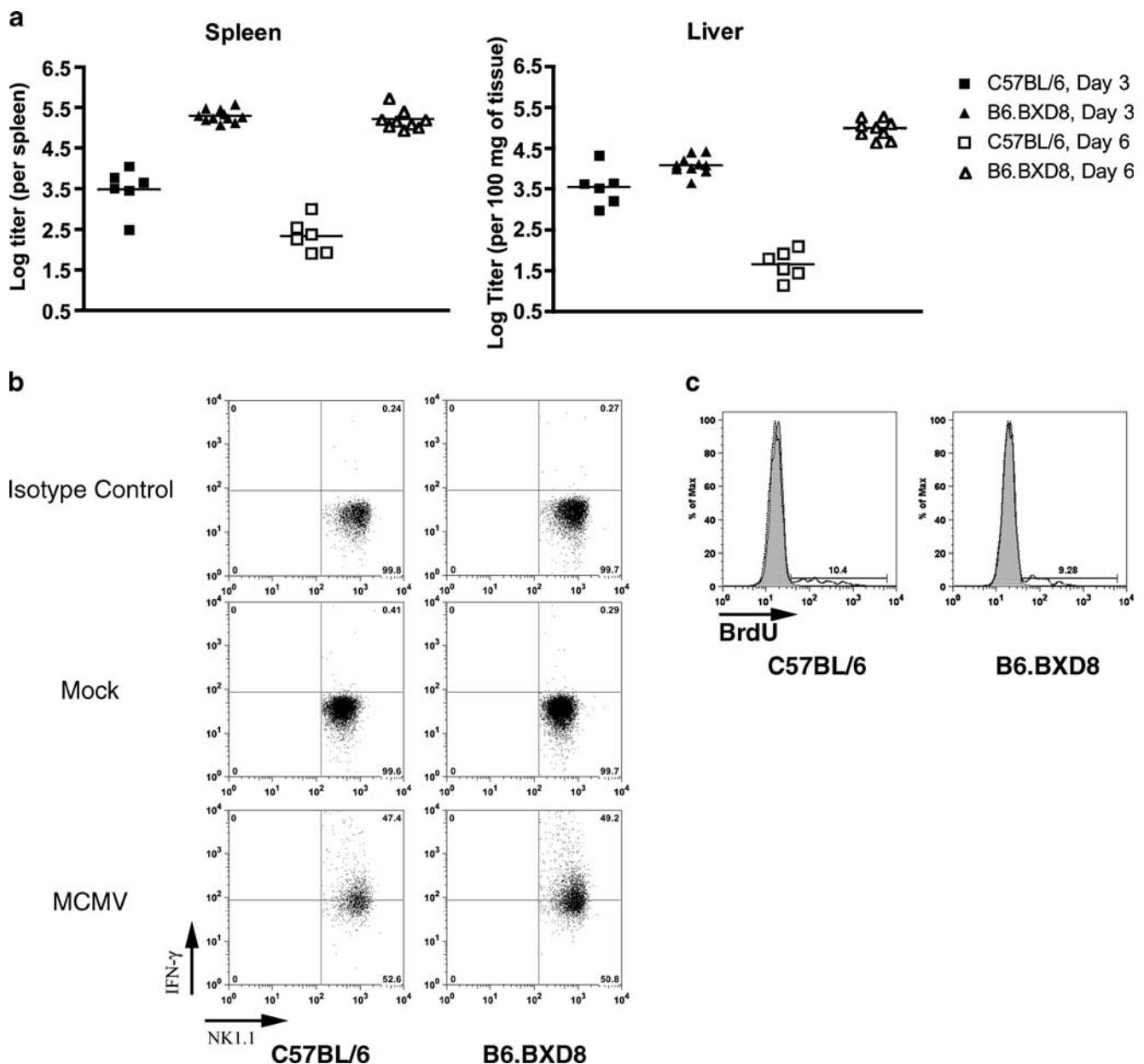


Fig. 4 MCMV infection in B6.BXD8. **a** Splenic and liver MCMV viral titers in C57BL/6 and B6.BXD8 mice. Each mouse was infected with 5×10^4 PFU of Smith strain MCMV intraperitoneally, and spleens and livers were harvested on day 3 post-infection. MCMV viral titers were quantified using methods described previously. **b** IFN- γ production in C57BL/6 and B6.BXD8 NK cells after MCMV infection. Mice were infected with 5×10^4 PFU of MCMV intraperitoneally, and spleens were harvested at 38 hours post-infection. Intracellular IFN- γ staining was performed on freshly isolated splenocytes following the manufacturer's protocol. The results shown were gated on the total NK cell population ($CD3^-/CD19^-/NK1.1^+$). The *upper two panels* show isotype controls for anti-IFN- γ antibody. The *middle two panels* are NK cells from mock-infected mice. The

lowest two panels show NK cells after MCMV infection. This result is representative of two independent experiments. **c** NK cell proliferation after MCMV infection. C57BL/6 and B6.BXD8 mice were infected with 5×10^4 PFU of MCMV and spleens harvested on day 2 post-infection. Three hours prior to splenocyte harvest, each mouse was intraperitoneally injected with 2 mg of BrdU, and subsequent intracellular BrdU staining was performed following the manufacturer's protocol. The histograms display total NK cell populations, defined as $CD3^-/CD19^-/NK1.1^+$. The *shaded curves* denote unstained controls, while the *dotted lines* denote cells stained with anti-BrdU antibody. The percentages of cells positive for anti-BrdU are quantified in the gates

We next sought to further delineate the MHC-independent contribution of *Ly49H* in controlling MCMV infection. We measured splenic and liver viral titers after intraperitoneally infecting both C57BL/6 and B6.BXD8 mice with Smith strain MCMV (Fig. 4a). Indeed, at day 3 post-MCMV infection, the splenic titers from B6.BXD8 mice were nearly two logs higher than those of C57BL/6 (mean log titers for C57BL/6 and B6.BXD8 were 3.5 and 5.3 PFU/spleen, respectively, $p < 0.0001$ by unpaired t test). Moreover, consistent with previous findings (Brown et al. 2001a), a small but statistically significant difference was found between hepatic viral titers between C57BL/6 and B6.BXD8 on day 3 post-infection ($p = 0.0046$ by unpaired t test). After infection with Smith strain MCMV, B6.BXD8 mice demonstrated a LD_{50} of 7×10^4 PFU, approximately threefold less than that for C57BL/6 (data not shown). Thus, these data indicate that *Ly49h* plays a significant role in C57BL/6 in controlling MCMV infection and rule out any epistatic effect by DBA/2 alleles in BXD-8.

During MCMV infection, we previously reported that NK cells are both non-specifically and specifically activated when considering *Ly49H* expression (Dokun et al. 2001). However, it was not clear if specific activation of NK cells through *Ly49H* contributed to non-specific activation. When we infected B6.BXD8 mice with MCMV, we found that B6.BXD8 NK cells express IFN- γ at similar levels as those of C57BL/6 NK cells at 38 hours post-MCMV infection (Fig. 4b). This finding indicated that the early, non-specific IFN- γ production occurs normally during MCMV infection in the absence of *Ly49h*. Another non-specific effect is the stimulation of NK cell proliferation early during MCMV infection. We observed no difference in the incorporation of BrdU in C57BL/6 versus B6.BXD8 splenocytes at 38 hours after MCMV infection (Fig. 4c). Thus, the “non-specific” (with respect to *Ly49H* expression) activation of NK cells does not require triggering through *Ly49H* itself (Dokun et al. 2001).

Previous genetic studies mapped the resistance to two other DNA viruses, HSV-1 and ectromelia virus, to NKC, but genes for host resistance have not been further elucidated (Brown et al. 2001a; Delano and Brownstein

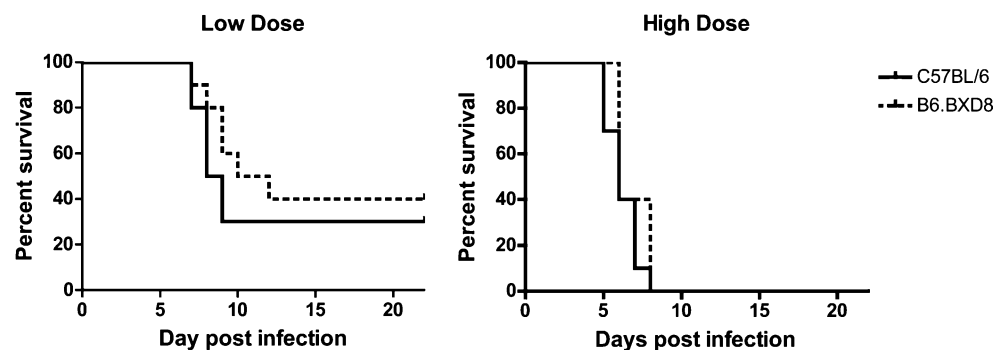
1995). We thus sought to evaluate whether the deletion of *Ly49h* on the C57BL/6 background altered host susceptibility to these viral infections. At both high and low doses of intraperitoneal HSV-1 infection, B6.BXD8 mice demonstrated similar rates of survival as C57BL/6 (Fig. 5). Similarly, infection with ectromelia virus resulted in comparable viral titers in C57BL/6 and B6.BXD8 mice on days 5, 6, and 7 post-infection as measured by quantitative PCR from two independent experiments (data not shown). Together, these data suggest that *Ly49h* does not account for NKC-linked genetic resistance to HSV or ectromelia virus infections.

Discussion

Natural killer cells play important roles in controlling viral infections, and how NK cells are able to recognize and control diverse viral pathogens with a limited repertoire of innate receptors is still poorly defined. In the case of C57BL/6 mice and control of MCMV viremia, we show here that the deletion of a single NK cell receptor, namely *Ly49h*, in the C57BL/6 genetic background greatly increased host susceptibility. This current paper further extends previous findings that *Ly49H*, a single NK activation receptor, plays a critical role in the control of MCMV infection in C57BL/6 mice because the present data exclude any epistatic genetic effect of DBA/2 alleles in the phenotype of BXD-8 mice. Taken together with previous work (Brown et al. 2001b; Lee et al. 2001, 2003), these data provide strong genetic evidence that *Ly49h* plays a major role in the ability of C57BL/6 mice to resist MCMV infection.

Although we were not able to pinpoint the genetic lesion in B6.BXD8, our novel strain demonstrated a specific deficiency of *Ly49H* at the protein level but intact expression of other examined receptors in the NKC region. *Ly49n* and *Ly49k*, two loci juxtaposing *Ly49h*, were previously identified as pseudogenes (McQueen et al. 1999). Their presence or absence is thus unlikely to account for the increased susceptibility of B6.BXD8 to MCMV, as

Fig. 5 Survival curves of C57BL/6 and B6.BXD8 mice after HSV infection. Mice were intraperitoneally inoculated with either low dose (3×10^6 PFU) or high dose (3×10^7 PFU) of strain 17 HSV-1. Mice were observed for survival for 22 days



compared to C57BL/6. Furthermore, while it is theoretically plausible that DBA/2 genes could have recombined into the *Ly49h* region in B6.BXD8 during the recombinant inbreeding process, this is very unlikely because a double crossover event would be needed in a very tight region. Regardless, B6.BXD8 has a specific *Ly49h* deletion on the C57BL/6 genetic background.

Interestingly, we previously detected non-stochastic expression of Ly49H with Ly49D on NK cells in normal B6 mice (Smith et al. 2000). These activation receptors both signal through the DAP12 signaling chain. One possible explanation for their co-expression is their interdependent transcriptional regulation. However, our current studies indicate that the absence of *Ly49h* did not appear to affect Ly49D expression, indicating that Ly49D expression is independent of Ly49H expression, raising the possibility that their tendency to be expressed with each other in normal mice may be related to their common requirement for DAP12.

Our current study found no gross defect with NK cell maturation markers, expression of other NKC-encoded receptors, or NK cell function in B6.BXD8 mice, indicating that Ly49H is not necessary for NK cell development. Importantly, unlike other Ly49 members which interact with host MHC class I molecules and modulate NK activation, Ly49H has no known endogenous ligand. Whether Ly49H plays roles other than antiviral protection against MCMV remains to be determined, and it is possible that Ly49H enables NK cells to recognize pathogens other than MCMV. However, in our current study, we found that B6.BXD8 and C57BL/6 mice are equally susceptible to HSV-1 and ectromelia virus, two pathogens whose resistance is encoded in the NKC region.

Our previous studies indicated that NK cells appear to undergo two phases of activation during MCMV infection, an early (days 1–2) “non-specific” (independent of Ly49H expression) and a later (days 3–6) “specific” phase whereby Ly49H⁺ NK cells undergo selective proliferation (Dokun et al. 2001). However, it was not clear if triggering through Ly49H itself contributed to the non-specific phase. Herein, we found that NK cells in B6.BXD8 mice have no defect in their non-specific responses, indicating that *Ly49h* is dispensable for early non-specific NK cell stimulation during MCMV infections.

Given the importance of Ly49 family in modulating NK cell functions, the novel strain B6.BXD8 may serve as a useful tool for further delineation of NK activation receptors. Previously, functional analysis of Ly49 activation receptors were derived from examining mice deficient or defective in the adaptor molecule DAP12, since activation Ly49 receptors do not contain intrinsic signaling motifs and signal downstream by association with the DAP12 adaptor molecule (Bakker et al. 2000; Kaifu et al. 2003; Smith et al.

1998). However, DAP12 can also associate with other activation receptors and is expressed in several different cell types (Bouchon et al. 2001), indicating that DAP12-deficient mice have broader defects. Therefore, B6.BXD8 will allow the evaluation of the endogenous function of Ly49H and indirectly other activation receptors with more precision.

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Note added in proof: The B6.BXD8 strain has been accepted for repository at The Jackson Laboratory and will be available through their facility.

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