# Inhibitory Ly49 Receptors on Mouse Natural Killer Cells

**Mark T. Orr and Lewis L. Lanier**

## Contents



**Abstract** The Ly49 receptors, which are expressed in a stochastic manner on subsets of murine natural killer (NK) cells, T cells, and other cells, are encoded by the *Klra* gene family and include receptors with either inhibitory or activating function. All of the inhibitory Ly49 receptors are characterized by an immunoreceptor tyrosine-based inhibitory motif in their cytoplasmic domain, which upon phosphorylation recruits tyrosine or lipid phosphatases to dampen signals transmitted through other activating receptors. Most of the inhibitory Ly49 receptors recognize polymorphic epitopes on major histocompatibility complex (MHC) class I proteins as ligands. Here, we review the polymorphism, ligand specificity, and signaling capacity of the inhibitory Ly49 receptors and discuss how these molecules regulate NK cell development and function.

M.T. Orr (✉) and L.L. Lanier (✉)
Department of Microbiology and Immunology and the Cancer Research Institute, University of California, San Francisco, CA 94143, USA
e-mail: mark.orr@ucsf.edu; lewis.lanier@ucsf.edu

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## 1 Introduction

The Ly49 receptors are type II C-type lectin-like glycoproteins encoded by a polygenic and polymorphic gene family designated *Klra*. The family includes genes encoding both inhibitory receptors that contain a single immunoreceptor tyrosine-based inhibitory motif (ITIM) and activating receptors that lack an intracellular signaling motif but instead non-covalently associate with the DAP10 adapter and/or the immunoreceptor tyrosine-based activating motif (ITAM)-containing adapter DAP12 (Orr et al. 2009; Smith et al. 1998). Expression of the activating receptors is restricted to natural killer (NK) cells, whereas inhibitory receptor family members are expressed predominantly by NK cells, but also by subsets of NKT cells, $CD4^+$ T cells, $CD8^+$ T cells, and myeloid cells (Vivier and Anfossi 2004). Although the Ly49 receptors are structurally related to the C-type lectins, they lack a $Ca^{2+}$-dependent carbohydrate recognition domain and do not bind to carbohydrate ligands. Instead, Ly49 receptors bind to major histocompatibility complex (MHC) class I and MHC class I-like proteins. Along with the MHC class I molecules, the Ly49 family is the fastest evolving gene family in rodents. Other species, including rats, cows, and horses, have multiple *Ly49* genes in their genomes, whereas cats, dogs, pigs, and orangutans possess only a single *Ly49* gene and in humans the only Ly49 locus, *KLRA1*, is a pseudogene (Gagnier et al. 2003). In humans, the functional counterpart of the Ly49 family is the killer cell immunoglobulin-like receptor (KIR) gene family, which encodes activating and inhibitory receptors that bind HLA class I molecules as ligands.

## 2 Ly49 Haplotypes and Ligands

The *Ly49* (*Klra*) gene family resides within the NK complex (NKC) on mouse chromosome 6 (Yokoyama and Plougastel 2003). Four haplotypes of the Ly49 family have been determined in inbred mice (Fig. 1) (Carlyle et al. 2008). Since MHC class I molecules are the predominant ligands for inhibitory Ly49 receptors, it is important to consider both the Ly49 receptor haplotype and MHC class I haplotype when studying NK cell functions. A minimal structure is conserved among all four Ly49 receptor haplotypes, three pairs of framework inhibitory receptors (*a* and *c*, *g* and *i*, and *e* and *q*) interspersed with a variable number of genes encoding inhibitory or activating receptors, as well as pseudogenes, most of which resemble activating receptor genes (Carlyle et al. 2008). Additionally, the *Klra2* (Ly49b) gene is retained in all strains, but lies outside of the *Ly49* gene cluster. The BALB/c ($H\text{-}2^d$) Ly49 haplotype, shared with AKR ($H\text{-}2^k$), DBA/2 ($H\text{-}2^d$), C3H/He ($H\text{-}2^k$), CBA/J ($H\text{-}2^k$), and A/J ($H\text{-}2^a$) stains of mice, contains only the additional *Ly49l* activating receptor gene and the *Ly49y* pseudogene (Proteau et al. 2004). C57BL/6 ($H\text{-}2^b$) mice contain fifteen *Ly49* genes, including genes encoding the six framework inhibitory receptors, two additional inhibitory

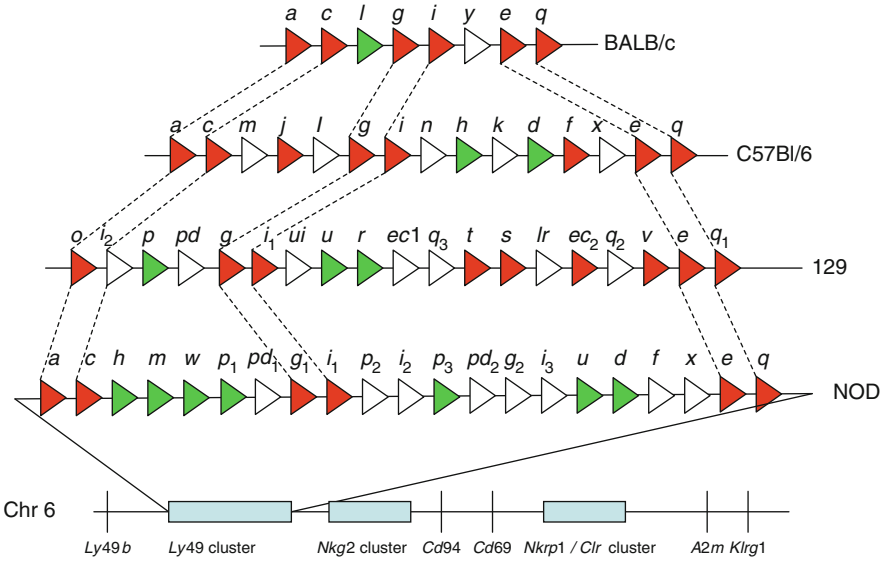


**Fig. 1** Schematic of the Ly49 locus in different haplotypes. Activating Ly49 family members are in *green*, inhibitory Ly49 family members are in *red*, and pseudogenes are in *white*. The locus is drawn telomeric to centromeric with the direction of transcription indicated. The conserved framework inhibitory receptors are connected via *dashed lines*

receptors (*Ly49f* and *j*), two activating receptors (*Ly49d* and *h*), and five pseudogenes (Brown et al. 1997; Depatie et al. 2000; McQueen et al. 1998, 1999; Smith et al. 1994; Takei et al. 1997; Wilhelm et al. 2002; Wong et al. 1991). The 129 (H-$2^b$) NKC shared with C57L/J (H-$2^b$), FVB/N (H-$2^q$), SJL/J (H-$2^{s2}$), and Ma/My (H-$2^k$) contains nineteen genes, three of which encode activating receptors (*Ly49r*, *u,* and *p*), three unique inhibitory receptors (*Ly49v*, *s*, and *t*), and seven pseudogenes (Makrigiannis et al. 2001, 2002). The framework *a* and *c* genes have been replaced with *o* and *i2* in the 129 (H-$2^b$) haplotype. The recently elucidated NOD/LtJ (H-$2^{g7}$) haplotype is the most diverse with seven genes encoding activating receptors (*Ly49h, m, w, p1, p3, u,* and *d*) and eight pseudogenes, in addition to the six framework inhibitory receptor genes (Belanger et al. 2008). Gene duplication and conversion are the major mechanisms generating the diversity of the Ly49 receptor repertoire, with the activating receptor genes being more recent in evolution and arising from inhibitory receptor genes (Abi-Rached and Parham 2005; Hao and Nei 2004). The extracellular domain of the activating Ly49D receptor is quite similar to that of the inhibitory Ly49A receptor, suggesting a common origin (Mehta et al. 2001a). The *Klra16* gene encoding Ly49P likely arose by a gene conversion event involving the exons encoding the transmembrane and intracellular domains of *Klra4* (Ly49d) and the extracellular domain of *Klra1* (Ly49a) (Makrigiannis et al. 1999). *Klra12* (Ly49l) and *Klra8* (Ly49h) likely arose from gene conversion involving exons derived from *Klra4* (Ly49d) and *Klra7* (Ly49g) or *Klra9*

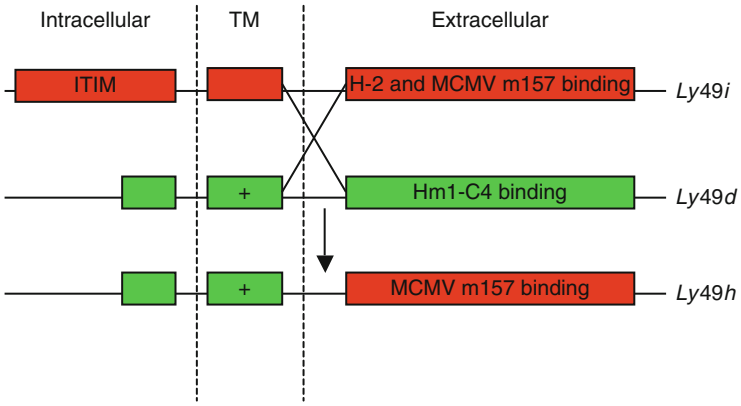


**Fig. 2** Generation of new Ly49 receptors by gene duplication and conversion. New Ly49 receptor family members may arise by recombination events between existing family members. *Ly49h* may have arisen by a duplication of *Ly49i* and a subsequent recombination between the exons encoding the extracellular MCMV m157 recognition domain and the exons encoding the intracellular and positively charged transmembrane (TM) domains of *Ly49d*

(Ly49i), respectively (Fig. 2). The close proximity within the genome and high-degree of sequence similarity in the *Klra* genes facilitates their very plastic and dynamic reorganization to generate genes with new ligand specificities and functions.

*Klra* genes encoding inhibitory Ly49 receptors are expressed largely in a stochastic, mono-allelic fashion on subsets of NK cells, although some individual NK cell clones demonstrate bi-allelic expression (Held and Kunz 1998; Held and Raulet 1997; Held et al. 1995). In contrast, the genes encoding activating Ly49 receptors are frequently expressed in a bi-allelic manner (Rouhi et al. 2009). In the steady-state Ly49 expression by individual mature NK cells is stable, but under certain environmental conditions new Ly49 receptors can be induced on mature NK cells (Dorfman and Raulet 1998); however, loss of Ly49 receptor expression on individual mature NK cell clones has not been observed (Orr et al. 2010).

The Ly49 receptors are expressed as disulfide-linked homodimers on the cell surface and recognize proteins encoded by various H-2 alleles and genes, as well as xenogeneic MHC class I and at least one virally encoded MHC class I-like molecule (Table 1). Recognition of MHC class I molecules requires β2-microglobulin and a peptide to be bound within the MHC class I groove. The identity of the peptide presented may sometimes affect Ly49 binding, although the details of this remain controversial. For example, replacement of all non-anchor residues in a peptide binding to H-2D$^{d}$ had no impact on recognition by the Ly49A$^{B6}$ receptor, but Ly49C$^{B6}$ was sensitive to changes in non-anchor peptide residues (Correa and Raulet 1995; Franksson et al. 1999; Hanke et al. 1999; Michaelsson et al. 2000). From a biological perspective, there is no evidence that the presence of "foreign" peptides

**Table 1** Ly49 receptors and their known receptors

| Common name | Gene name | Strain haplotype | Function | Ligands | Ligand excluded |
|---|---|---|---|---|---|
| Ly49A | *Klra1* | B6 | Inhibiting | H-2D$^{d}$, D$^{k}$, D$^{p}$, H-2$^{f, q, r, s, v}$ | H-2K$^{d}$, K$^{k}$, Ld, H-2$^{b}$ |
| Ly49B | *Klra2* | B6 | Inhibiting | | H-2$^{b, d, k, f, q, r, s, v}$ |
| Ly49C | *Klra3* | B6 | Inhibiting | H-2K$^{b}$, K$^{d}$, D$^{d}$, D$^{k}$, H-2$^{f, q, r, s, v}$ | H-2D$^{b}$, L$^{d}$ |
| Ly49D | *Klra4* | B6 | Activating | H-2D$^{d}$, Hm1-C4 | |
| Ly49E | *Klra5* | B6 | Inhibiting | Urokinase plasminogen activator | H-2$^{b, d, k, f, q, r, s, v}$ |
| Ly49F | *Klra6* | B6 | Inhibiting | H-2K$^{d}$ | H-2D$^{d}$, L$^{d}$, H-2$^{b, k, f, q, r, s, v}$ |
| Ly49G2 | *Klra7* | B6 | Inhibiting | H-2D$^{d}$ | H-2K$^{d}$, L$^{d}$, H-2$^{b, k, f, q, r, s, v}$ |
| Ly49H | *Klra8* | B6 | Activating | MCMV m157 | |
| Ly49I | *Klra9* | B6 | Inhibiting | H-2K$^{b}$, D$^{d}$, K$^{d}$, K$^{k}$, H-2$^{q, r, s, v}$ | MCMV m157, H0-L$^{d}$, D$^{b}$, H-2$^{f}$ |
| Ly49J | *Klra10* | B6 | Inhibiting | | |
| Ly49Q | *Klra17* | B6 | Inhibiting | H-2K$^{b}$ | H-2$^{a, d, k, q}$ |
| Ly49B | *Klra2* | 129 | Inhibiting | | |
| Ly49E | *Klra5* | 129 | Inhibiting | | |
| Ly49G2 | *Klra7* | 129 | Inhibiting | H-2D$^{d}$, D$^{k}$, K$^{d}$, D$^{b}$ | H-2K$^{b}$, K$^{k}$, L$^{d}$ |
| Ly49I | *Klra9* | 129 | Inhibiting | H-2D$^{k}$, K$^{b}$, K$^{d}$, K$^{d}$ MCMV m157 | H-2D$^{b}$, D$^{d}$, L$^{d}$ |
| Ly49I2 | *Klra10* | 129 | Inhibiting | | |
| Ly49O | *Klra15* | 129 | Inhibiting | H-2D$^{b}$, D$^{d}$, D$^{k}$, L$^{d}$ | H-2K$^{b}$, K$^{d}$, K$^{k}$ |
| Ly49P | *Klra16* | 129 | Activating | H-2D$^{k}$ in conjunction with MCMV m04 | |
| Ly49Q | *Klra17* | 129 | Inhibiting | | |
| Ly49R | *Klra18* | 129 | Activating | | |
| Ly49S | *Klra19* | 129 | Inhibiting | | H-2$^{b, d, k}$ |
| Ly49T | *Klra20* | 129 | Inhibiting | | H-2$^{b, d, k}$ |
| Ly49U | *Klra21* | 129 | Activating | | |
| Ly49V | *Klra22* | 129 | Inhibiting | H-2D$^{b}$, D$^{d}$, D$^{k}$, K$^{b}$, K$^{d}$, K$^{k}$, L$^{d}$ | |
| Ly49A | *Klra1* | BALB/c | Inhibiting | H-2D$^{d}$ | |
| Ly49B | *Klra2* | BALB/c | Inhibiting | | |
| Ly49C | *Klra3* | BALB/c | Inhibiting | H-2K$^{b}$, K$^{d}$, D$^{d}$ D$^{k}$, H-2$^{f, q, r, s, v}$ | H-2D$^{b}$, L$^{d}$ |
| Ly49E | *Klra5* | BALB/c | Inhibiting | | |
| Ly49G2 | *Klra7* | BALB/c | Inhibiting | H-2D$^{d}$, D$^{k}$ | |
| Ly49I | *Klra9* | BALB/c | Inhibiting | | |
| Ly49L | *Klra12* | BALB/c | Activating | | |
| Ly49Q | *Klra17* | BALB/c | Inhibiting | | |
| Ly49A | *Klra1* | NOD | Inhibiting | | |
| Ly49B | *Klra2* | NOD | Inhibiting | | |
| Ly49C | *Klra3* | NOD | Inhibiting | | |
| Ly49D | *Klra4* | NOD | Activating | | |
| Ly49E | *Klra5* | NOD | Inhibiting | | |
| Ly49G2 | *Klra7* | NOD | Inhibiting | | |
| Ly49H | *Klra8* | NOD | Activating | | |
| Ly49I | *Klra9* | NOD | Inhibiting | | |
| Ly49M | *Klra13* | NOD | Activating | | |

(*continued*)

**Table 1** (continued)

| Common name | Gene name | Strain haplotype | Function | Ligands | Ligand excluded |
|---|---|---|---|---|---|
| Ly49P1 | | NOD | Activating | | |
| Ly49P3 | *Klra16* | NOD | Activating | | |
| Ly49Q | *Klra17* | NOD | Inhibiting | | |
| Ly4U | *Klra21* | NOD | Activating | | |
| Ly4W | *Krla23* | NOD | Activating | | |

in the H-2 groove would be preferentially recognized over "self" peptides by Ly49 receptors.

Affinities for MHC class I as measured by tetramer binding and cell adhesion vary with the Ly49 receptor and allele. Proteins encoded by different alleles of H-2 bind with different strength to the same Ly49 receptor, resulting in a wide array of specificities and affinities between Ly49 receptors and MHC class I molecules. The MHC class I specificity for the C57BL/6 inhibitory receptors Ly49A, C, I, and G2 have been the most extensively studied. The Ly49A receptor encoded by the C57BL/6 allele of the *Klra1* gene (designated Ly49A$^{B6}$) binds H-2D$^{d}$, D$^{k}$, and D$^{p}$, as well as H-2 from f, q, r, s, and v haplotypes, but not H-2$^{b}$, K$^{d}$, K$^{k}$, or L$^{d}$ (Takei et al. 1997; Hanke et al. 1999; Daniels et al. 1994; Kane 1994; Karlhofer et al. 1992; Olsson-Alheim et al. 1999). Ly49A$^{B6}$ has the highest affinity for H-2$^{d}$ with a $K_D$ of ~10 μM, followed in order of decreasing affinity by H-2$^{r}$, H-2$^{k}$, H-2$^{q}$, and H-2$^{s}$ (Jonsson et al. 2010; Natarajan et al. 1999). The allele encoding the Ly49A receptor in BALB/c mice (Ly49A$^{BALB}$), which varies from Ly49A$^{B6}$ by four amino acids, also binds H-2D$^{d}$, but with lower affinity than the Ly49A$^{B6}$ receptor (Mehta et al. 2001b). Ly49C$^{B6}$ and Ly49C$^{BALB}$, which also differ by four amino acids, both bind H-2K$^{b}$, K$^{d}$, D$^{d}$, and D$^{k}$ in addition to H-2 from the f, q, r, s, and v haplotypes, but not H-2D$^{b}$ or H-2L$^{d}$ (Hanke et al. 1999; Brennan et al. 1996; Lian et al. 1999; Raulet et al. 1997). Ly49I$^{B6}$ binds H-2K$^{b}$, D$^{d}$, K$^{d}$, and K$^{k}$, as well as H-2 from q, r, s, and v haplotypes, but not H-2$^{f}$, H-2L$^{d}$, or H-2D$^{b}$ (Hanke et al. 1999). Ly49I$^{129}$ (the allele of *Klra9* in 129/J mice) recognizes the virally encoded m157 MHC class I-like molecule encoded by the Smith strain of mouse cytomegalovirus (MCMV), which is also recognized by the activating Ly49H$^{B6}$, but not by Ly49I$^{B6}$ or Ly49I$^{BALB}$ (Arase et al. 2002; Smith et al. 2002). Ly49G2$^{B6}$ binds H-2D$^{d}$, but not H-2K$^{d}$, H-2L$^{d}$, or H-2 from b, k, f, q, r, s or v haplotypes (Hanke et al. 1999; Johansson et al. 1998; Mason et al. 1995). Ly49G2$^{BALB}$ binds H-2D$^{b}$ with higher affinity than Ly49G2$^{B6}$, and also binds H-2D$^{k}$ (Silver et al. 2002). Importantly, neither Ly49A$^{B6}$ nor Ly49G2$^{B6}$ have any measurable affinity for H-2$^{b}$ proteins and thus do not recognize self-MHC class I in C57BL/6 mice.

Whereas the interactions between several inhibitory Ly49 receptors expressed on NK cells and classical MHC class I molecules have been extensively documented and their biological consequences well defined, the ligands of other members of the Ly49 family that are expressed on other cell types are not well established. Ly49F$^{B6}$ binds weakly to H-2K$^{d}$, but not H-2D$^{d}$, H-2L$^{d}$, or H-2 from b, k, f, q, r, s,

or v haplotypes (Hanke et al. 1999). Ly49Q, which is expressed on myeloid cells, but not NK cells or T cells, binds H-2K$^{b}$, but not H-2 from a, d, k, or q haplotypes (Sasawatari et al. 2010; Tai et al. 2007, 2008; Toyama-Sorimachi et al. 2004). Similarly, Ly49B, which is expressed by macrophages, does not bind H-2 molecules of the b, d, k, f, q, r, s, or v haplotypes (Hanke et al. 1999; Gays et al. 2006). Many transcripts of *Klra10* (Ly49j) lack a transmembrane and thus may encode for an intracellular protein and the specificity of Ly49J has not been determined (McQueen et al. 1999). Ly49E is expressed on some γδ T cells and fetal NK cells, but is rarely expressed on adult NK cells (Fraser et al. 2002; Van Beneden et al. 2001, 2002). Ly49E, which does not bind H-2 molecules of the b, d, k, f, q, r, s, or v haplotypes, recognizes cells expressing the urokinase plasminogen activator protein, although an interaction between Ly49E and urokinase plasminogen activator protein has not been shown directly (Van Den Broeck et al. 2008). The functional importance of this interaction remains unknown.

The ligand specificities of the Ly49 receptors expressed by 129/J mice have also been investigated using H-2$^{b}$, H-2$^{d}$, and H-2$^{k}$ tetrameric reagents (Makrigiannis et al. 2001). Ly49V$^{129}$ bound all H-2 tetramers tested: D$^{b}$, D$^{d}$, D$^{k}$, K$^{b}$, K$^{d}$, K$^{k}$, and L$^{d}$. Despite being 96% identical at the amino acid level to Ly49G2$^{B6}$, Ly49G2$^{129}$ displays much broader reactivity to H-2 alleles than Ly49G2$^{B6}$, binding H2-D$^{d}$, D$^{k}$, K$^{d}$, and D$^{b}$. Ly49I$^{129}$ displayed a similar affinity for H-2 as Ly49I$^{B6}$ (96% identity), binding H2-D$^{k}$, K$^{b}$, K$^{d}$, and K$^{d}$. Ly49O$^{129}$ shares the highest identity with Ly49A$^{B6}$ (93%) and recognizes H2-D$^{b}$, D$^{d}$, D$^{k}$, and L$^{d}$. The inhibitory receptors Ly49S$^{129}$ and Ly49T$^{129}$ did not bind any of the tetramers tested. Because of the different *Ly49* gene content and differing Ly49 receptor affinities for H-2 alleles in C57BL/6 mice compared with 129/J mice, the use of 129/J embryonic stem cells to ablate alleles within or near the *Klra* loci may lead to difficulties in interpreting results if the gene-deficient mice generated with 129/J embryonic stem cells are backcrossed onto the C57BL/6 background because they will typically retain the 129/J NKC genomic region. The affinities of the Ly49 receptors encoded by genes of the NOD/LtJ genetic background have not been reported.

Some activating members of the Ly49 receptor family also bind MHC class I and MHC class I-like molecules. The activating receptor Ly49D binds the Hm1-C4 MHC class I molecule from hamsters, accounting for Ly49D-mediated lysis of CHO cells by NK cells from C57BL/6 mice (Merck et al. 2009). NK cells expressing Ly49D recognize target cells expressing H-2D$^{d}$ but not other MHC class I molecules, although direct binding of Ly49D to H-2D$^{d}$ has not been reported (George et al. 1999a, b). Ly49H$^{B6}$ directly recognizes the MCMV-encoded m157 protein expressed on the surface of infected cells, but does not bind to any H-2 ligand (Arase et al. 2002; Smith et al. 2002; Adams et al. 2007). Ly49P$^{Ma/My}$ recognition of MCMV-infected cells is dependent on the expression of the viral m04 protein and H-2D$^{k}$, but not H-2K$^{k}$ or other H-2 alleles (Desrosiers et al. 2005; Kielczewska et al. 2009). MCMV m04 binds to MHC class I molecules and traffics to the cell surface and may modify the conformation of H-2D$^{k}$ allowing recognition by Ly49P (Hengel et al. 1999).

## 3 Inhibitory Signaling Events

All inhibitory receptors in the Ly49 family express in their cytoplasmic domains an ITIM, which is characterized by the signature sequence, (I/L/V/S)xYxx (L/V) (where x represents any amino acid, and slashes separate alternative amino acids that may occupy a given position). The mechanisms by which NK cell inhibitory receptors abrogate NK cell activation have been best worked out for the human inhibitory KIR family. These signaling pathways are thought to be similar for inhibitory Ly49 receptors, although this may not always be the case (Lanier 2008; Long 2008; MacFarlane and Campbell 2006). When the inhibitory receptors on NK cells bind to their MHC class I ligands on potential target cells, the ITIMs are phosphorylated by Src family kinases including Lck (Fig. 3) (Binstadt et al. 1996). The SH2-domain-containing protein tyrosine phosphatases 1 and 2 (SHP-1 and SHP-2) are recruited to the phosphorylated ITIMs at the immunological synapse (Daws et al. 1999; Eriksson et al. 1999a; Fassett et al. 2001; Fry et al. 1996; Mason et al. 1997; Nakamura et al. 1997; Olcese et al. 1996; Vyas et al. 2004; Vyas et al. 2001, 2002; Burshtyn et al. 1996). SHP-1 and -2 are normally in an inactive conformation with the SH2 domain bound to the catalytic domain. Binding of phosphorylated ITIMs releases the SH2 domain, allowing SHP-1 and -2 to become catalytically active (Hof et al. 1998; Tonks and Neel 1996). SHP-1 and 2 dephosphorylate different substrates, and thus likely have differing, non-redundant roles in NK cell inhibition (Mishra et al. 2002; Yang et al. 1998). The motheaten viable mutation of SHP-1 is sufficient to abrogate Ly49A, Ly49C, and Ly49I inhibition of NK cell activation; thus, SHP-1 may be the primary mediator of inhibition by Ly49 receptors (Orr et al. 2010; Nakamura et al. 1997). Although a number of proteins including Src family kinases, PLCγ, ZAP70, Vav, SLP76, LAT, Grb2, and PI3K are dephosphorylated when inhibitory receptors are triggered, it is unclear whether all of these are direct substrates of the recruited phosphatases or represent downstream abrogation of activation (Binstadt et al. 1996, 1998; Palmieri et al. 1999; Stebbins et al. 2003; Valiante et al. 1996). However, Vav1 is a direct target of SHP-1 and is a critical target of dephosphorylation upon inhibitory receptor ligation (Stebbins et al. 2003). Vav1 is phosphorylated upon NK cell activating receptor triggering and is necessary for cytoskeletal rearrangements, secretion of cytotoxic granules, and release of effector cytokines and chemokines including IFN-γ, TNF, MIP1α, and RANTES (Long 2008). The SH2-domain-containing inositol polyphosphate 5′ phosphatase-1 (SHIP-1) is also recruited to the phosphorylated ITIMs of Ly49 receptors, but not human KIRs (Daws et al. 1999; Gupta et al. 1997; Wang et al. 2002). SHIP-1 dephosphorylates PI-3,4,5-$P_3$ to PI-3,4-$P_2$, thus abrogating $Ca^{2+}$-dependent signaling. Over-expression of SHIP-1 inhibits CD16-mediated antibody-dependent cellular cytotoxicity (Galandrini et al. 2001).

In human NK cells, HLA class I binding to inhibitory KIRs induces phosphorylation of Crk, and disruption of the Crk-cCbl-C3G-p130CAS complex (Peterson and Long 2008). Whether a similar mechanism of inhibitory signaling is active upon inhibitory Ly49 triggering remains to be determined. β-arrestin has also been

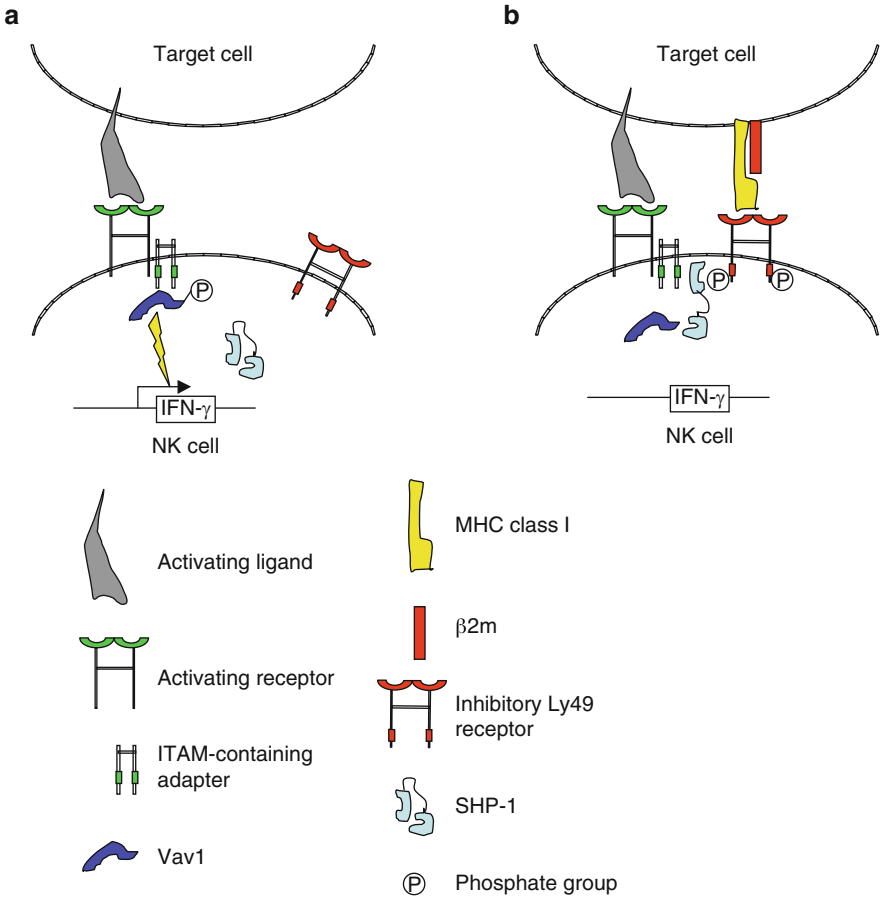


**Fig. 3** Signaling by inhibitory Ly49 receptors. (**a**) Upon engagement with cognate ligands on target cells activating receptors on NK cells signal via ITAM-containing adapter proteins or DAP10, an adapter protein containing an YINM motif, to phosphorylate Vav1, resulting in NK cell activation. (**b**) Engagement with MHC class I on target cells recruits inhibitory Ly49 receptors on the NK cell to the immunological synapse and results in phosphorylation of the ITIM domains. SHP-1, normally in a closed, inactive state, binds to the phosphorylated ITIM domains via the SH2 domain, freeing the phosphatase domain to dephosphorylate Vav1 and dampen NK cell activation

implicated in the recruitment of SHP-1 and 2 to the phosphorylated ITIMs of KIR (Yu et al. 2008). Whether β-arrestin is necessary for inhibitory Ly49 function remains to be determined; however, NK cells from β-arrestin-deficient mice displayed increased cytotoxicity toward NK susceptible and resistant targets and these mice controlled MCMV infection better than wildtype mice in an NK cell-dependent manner (Yu et al. 2008).

The strength of the inhibitory signal varies directly with the affinity of the inhibitory receptor for the MHC class I ligand. For example, the affinity of

Ly49A for H-2D$^d$ is much stronger than for H-2D$^k$, and Ly49A ligation of H-2D$^d$ is more inhibitory than ligation of H-2D$^k$ (Hanke et al. 1999; Jonsson et al. 2010). Simultaneous engagement of multiple inhibitory Ly49 receptors increases the strength of inhibition (Hanke and Raulet 2001). Interestingly, simultaneous engagement of inhibitory Ly49 receptors by MHC class I by one potential target cell does not prevent the same NK cell from being activated by and lysing a second target cell that does not engage an inhibitory receptor (Eriksson et al. 1999b). This implies that the inhibitory signaling events must be restricted to one subcellular location near the cells surface of the NK cell, while allowing another site on the cell surface interacting with another target cell to mediate NK cell activation unimpaired.

In addition to binding MHC class I in *trans* on other cells, Ly49 receptors also interact in *cis* with MHC class I on the same NK cell surface membrane. This *cis* interaction impedes the binding of some antibodies against the Ly49 receptors leading to an apparent "down-regulation" of these receptors by *cis* interactions (Scarpellino et al. 2007). These *cis* interactions sequester inhibitory receptors away from the immunologic synapse, reducing the inhibitory capacity of Ly49 receptors that bind to self-MHC class I (Chalifour et al. 2009; Doucey et al. 2004). Additionally, *cis* interactions require Ly49 receptors to adopt a different conformation from the one used to bind MHC class I in *trans* and may result in different intracellular signaling events that do not result in NK cell inhibition (Doucey et al. 2004; Back et al. 2009).

## 4 Educational Impact of Ly49 Receptors on NK Cells

Ly49 receptors are expressed in a variegated, overlapping manner resulting in many subsets of NK cells that express different constellations of inhibitory Ly49 receptors or in some cases no Ly49 receptors on some NK cells (Raulet et al. 1997; Kubota et al. 1999). For example, in C57BL/6 mice 30–50% of mature NK cells lack Ly49C and Ly49I and thus do not recognize self-MHC class I via Ly49 inhibitory receptors. Although these cells are phenotypically indistinguishable from cells that express Ly49C and/or Ly49I, they are less responsive to triggering through their activating receptors (Fernandez et al. 2005). NK cells lacking Ly49C and Ly49I are also impaired in their ability to acutely reject MHC class I-deficient bone marrow (Fernandez et al. 2005). Similarly, responsiveness of Ly49A$^{B6}$ single-positive NK cells correlated with expression of H-2D$^d$, which is a ligand of Ly49A, but not H-2$^b$, which is not ligated by Ly49A$^{B6}$ (Kim et al. 2005). NK cells from MHC class I-deficient mice are hyporesponsive to activation through multiple activating receptors and fail to reject MHC class I-deficient bone marrow (Fernandez et al. 2005; Kim et al. 2005; Hoglund et al. 1991). Responsiveness can be restored to the Ly49C subset of NK cells by from MHC class I deficient mice transgenic expression of H-2K$^b$ in MHC class I-deficient mice (Kim et al. 2005). Thus, the interaction between self-MHC class I and inhibitory Ly49 receptors engenders NK

cell responsiveness and has been termed "licensing" or "arming" of NK cells by inhibitory Ly49 receptors (Brodin et al. 2009a).

Expression of multiple self-reactive inhibitory receptors increases the responsive capacity of NK cells, such that NK cells expressing both Ly49C and Ly49I are more responsive than either Ly49C or Ly49I single-positive NK cells (Brodin et al. 2009b; Joncker et al. 2009). The affinity for self-MHC class I also affects the functional responsiveness of the NK cell. For example, in Ly49A$^{+}$ NK cells the high affinity H-2D$^{d}$ ligand engenders more responsive capacity than low affinity ligands such as H-2$^{s}$ (Jonsson et al. 2010). Expression of the inhibitory CD94-NKG2A inhibitory receptor that binds the MHC class Ib molecule Qa-1 also enhances NK cell responsiveness (Fernandez et al. 2005). Mutations to the ITIM domain of Ly49 inhibitory receptors abrogate NK cell licensing or arming, indicating that licensing depends on signaling by the inhibitory Ly49 receptor (Kim et al. 2005). Licensing occurs independently of SHP-1 and SHIP signaling, suggesting other signaling pathways must be engaged downstream of ITIM phosphorylation to engender responsiveness (Orr et al. 2010; Kim et al. 2005). The hyporesponsiveness of NK cells from mice lacking surface expression of MHC class I has been used to explain why NK cells from mice lacking β2-microglobulin, TAP-1, or H-2K and H-2D heavy chains do not exert overt autoimmunity (Hoglund et al. 1998; Liao et al. 1991; Ljunggren et al. 1994), and why NK cells from *B2m*$^{-/-}$ mice fail to lyse *B2m*$^{-/-}$ T cell blasts (Hoglund et al. 1991).

In mixed bone marrow chimeras containing MHC class I-sufficient and deficient hematopoetic cells, NK cells of both genotypes are hyporesponsive against MHC class I-deficient target cells and the chimerism is stable (Wu and Raulet 1997). This suggests that licensing is not mediated by *cis* interactions with MHC class I expressed on the NK cells, as has been recently suggested (Chalifour et al. 2009). A transgenic mouse model expressing H-2D$^{d}$ on only a subset of cells (mosaic expression) also renders NK cells hypofunctional due to the presence of a large number of cells not expressing the H-2D$^{d}$ transgene (Johansson et al. 1997). These results suggest that the hyporesponsiveness is dominant and inducible by lack of MHC class I interactions with inhibitory Ly49 receptors. MHC class I-deficient bone marrow is rejected by NK cells in MHC class I-sufficient recipients, suggesting that bone marrow cells express at least one activating ligand that if not opposed by MHC class I engagement of inhibitory receptors is sufficient to activate NK cells. Thus, chronic exposure to activating ligands unopposed by inhibitory receptors for self-MHC class I may "disarm" NK cells including Ly49C$^{-}$ and Ly49I$^{-}$ NK cells in C57BL/6 mice or all NK cells in MHC class I-deficient mice (Gasser and Raulet 2006). This would be consistent with the hyporesponsiveness of NK cells in the mixed bone marrow chimeric mice containing MHC class I-sufficient and deficient hematopoetic cells. It is unclear if lack of expression by MHC class I by a particular cell type drives disarming of NK cells or whether it is simply an overwhelming number of MHC class I-deficient cells that express one or more activating ligands. Responsiveness of disarmed or unlicensed NK cells can be restored in a variety of ways including culture in IL-2, stimulation with high doses of IL-12 and IL-18, or in vivo by infection with *Listeria monocytogenes* or MCMV (Orr et al. 2010;

Fernandez et al. 2005; Kim et al. 2005; Sun and Lanier 2008; Yokoyama and Kim 2006).

## 5 Inhibitory Ly49 Receptors and Viral Infections

Inhibitory receptors regulate NK cell responses at the level of detection of alterations in MHC class I expression. $CD8^+$ T cells are activated by T cell receptor engagement of cognate MHC class I:peptide ligands. Inhibition of MHC class I expression is a common immune evasion strategy employed by many viruses, including herpesviruses, adenovirus, and HIV (Tortorella et al. 2000). Loss of MHC class I on transformed cells is also a frequent event during malignancy (Marincola et al. 1994). Both viral and transformation-induced loss of MHC class I renders target cells invisible to recognition and clearance by $CD8^+$ cytotoxic T cells. However, the loss of self-MHC class I, termed "missing self", removes the inhibitory signals provided by Ly49 receptors on NK cells thereby allowing NK cells to detect and eliminate infected or transformed cells that express one or more ligands for activating NK cell receptors (Hoglund et al. 1991; Liao et al. 1991; Bix et al. 1991; Karre et al. 1986; Lanier 2005).

To date there remains little in vivo evidence addressing the significance of missing self-recognition to NK cell control of viral infection. MCMV is the most well studied example of viral control by NK cells in mouse models (Orr et al. 2010; Arase et al. 2002; Bukowski et al. 1983; Dokun et al. 2001; Sun et al. 2009). Although MCMV encodes two proteins that impede expression of MHC class I on the surface of infected cells, there are no reports of this enhancing NK cell control of infection in vivo (Doom and Hill 2008). Conversely, NK cell activating receptors play a critical role in the activation of NK cells and elimination of MCMV-infected cells. $Ly49H^{B6}$ ligation by the MCMV-encoded m157 glycoprotein that is expressed on the cell surface of infected cells activates NK cells and is necessary for NK cell control of MCMV infection (Arase et al. 2002; Smith et al. 2002). During MCMV infection $Ly49H^+$ NK cells proliferate extensively after recognition of the cognate ligand MCMV-m157 (Dokun et al. 2001). Ly49C and/or Ly49I receptors restrain this Ly49H-driven proliferation by interacting with self-MHC class I via inhibitory signaling through SHP-1 (Orr et al. 2010). Consequently, licensed $Ly49C/I^+$ $Ly49H^+$ NK cells make very little contribution to viral control, rather it is the unlicensed or disarmed $Ly49C/I^-$ $Ly49H^+$ NK cells that control MCMV replication (Orr et al. 2010). Thus, in the case of MCMV infection where contact with the infected cells is required for NK cells to mediate immunity, Ly49C/I-mediated inhibition of NK cell functions overrides the responsive benefit gained by licensing, whereas the unlicensed or disarmed NK cells, likely activated by the inflammatory milieu associated with infection, are competent to respond to MCMV-infected cells, unimpeded by inhibitory Ly49 receptor signaling. It is possible that these competent NK cells that are not inhibited by self-MHC class I mediate collateral damage by attacking uninfected cells expressing activating ligands.

However, during MCMV infection Ly49H$^+$ NK cells upregulate the inhibitory receptor KLRG1, which binds cadherins expressed on host cells, thus non-MHC class I restricted inhibitory receptors may prevent auto-aggression by these cells (Sun et al. 2009; Grundemann et al. 2006; Ito et al. 2006; Robbins et al. 2004). The decrease in the frequency of NK cells in C57BL/6 mice expressing Ly49C and/or Ly49I that occurs during MCMV infection has also been observed during other infections including lymphocytic choriomeningitis virus, vaccinia, and mouse hepatitis virus, and is thus not unique to MCMV infection (Orr et al. 2010; Daniels et al. 2001).

## 6 Inhibitory Ly49 Receptors in Transplantation and Malignancy

In bone marrow transplantation, NK cells play an important role in preventing graft-versus-host disease (GVHD), while still conferring a beneficial graft-versus-leukemia (GVL) effect (Glass et al. 1996; Asai et al. 1998). GVHD results when donor allogeneic T cells included in the graft bone marrow are activated by recipient antigen-presenting cells (APCs) displaying recipient MHC antigens. GVHD can be prevented by depletion of donor T cells from the bone marrow graft, but this often results in leukemia relapse (Shlomchik et al. 1999). Co-transferred alloreactive donor NK cells are able to kill recipient APCs, thereby preventing GVHD and enhancing engraftment of the donor bone marrow (Ruggeri et al. 2002). Simultaneously, these NK cells kill residual allogeneic host leukemic cells, thus increasing disease-free survival. NK cells lacking inhibitory Ly49 receptors for host H-2 mediate both the GVL effects and killing of host APCs to prevent GVHD. Co-transfer of these uninhibited donor NK cells allows for transfer of 20 times more donor T cells, which speeds up the reconstitution of the immune system, limiting fatal infections after transplantation (Ruggeri et al. 2002). NK cell prevention of GVHD can also be enhanced by siRNA knockdown of inhibitory Ly49 receptors that recognize the recipient MHC class I (Cao et al. 2009). In the case of MHC class I-matched donor bone marrow transferred into lethally irradiated hosts, such as BALB/c (H-2$^d$) mice receiving B10.D2 (H-2$^d$) bone marrow, host APCs prime donor T cells against minor histocompatibility antigens, resulting in delayed GVHD. Adoptive transfer of Ly49C/I$^+$ Ly49G2$^-$ NK cells from B10.D2 mice limited GVHD, whereas transfer of equal numbers of Ly49C/I$^-$ Ly49G2$^+$ NK cells from B10.D2 had little effect because they are inhibited by recipient H-2$^d$ (Lundqvist et al. 2007). Thus, although Ly49C $^{B6}$ and Ly49I$^{B6}$ recognize H-2$^d$, H-2$^d$-mediated inhibition appears stronger for Ly49G2 than for Ly49C or Ly49I. Similarly, in a model of lung metastases, adoptively transferred Ly49C/I$^+$ Ly49G2$^-$ NK cells were more efficient than a similar number of Ly49C/I$^-$ Ly49G2$^+$ NK cells in preventing the growth of the renal carcinoma cell line RENCA, which expresses H-2$^d$, in BALB/c mice (Lundqvist et al. 2007).

"Missing self" recognition can be mimicked by blocking Ly49 interactions with MHC class I by using Ly49 specific antibodies. Blockade of Ly49C and/or Ly49I by using $F(ab')_2$ fragments of the 5E6 monoclonal antibody enhanced endogenous NK cell-mediated control of the H-$2^b$ leukemic cell line C1498 in vivo in C57BL/6 mice (Koh et al. 2001). Moreover, adoptive transfer of IL-2-activated NK cells enhanced control of the C1498 tumor in vivo, and this was further enhanced by blocking the Ly49C and Ly49I receptors (Koh et al. 2001). C57BL/6 NK cells are also able to purge C1498 leukemic cells from bone marrow prior to infusion, resulting in an increase in leukemia-free survival of irradiated recipient mice. Blocking Ly49C and Ly49I during this conditioning period further reduced the leukemic burden, increasing survival of recipient mice without damage to the normal bone marrow cells (Koh et al. 2002). Co-culture of bone marrow containing leukemia cells with NK cells from H-$2^d$ donors more efficiently eliminated leukemic cells than NK cells from H-$2^b$ donors, resulting in increased leukemia-free survival of irradiated recipients. Again, this was further enhanced by blocking Ly49C and Ly49I during the ex vivo conditioning period (Koh et al. 2003). Although it is unclear why H-$2^d$ NK cells were more efficacious than H-$2^b$ NK cells, it is possible that Ly49C/$I^-$ NK cells expressing Ly49A and/or Ly49G2 are licensed by the donor H-$2^d$, but not donor H-$2^b$, and are not inhibited by H-$2^b$ on the leukemic cells and thus are more efficient at killing the leukemia targets.

In contrast to hematopoietic malignancies, NK cells are less efficient at controlling solid organ tumors (Yu et al. 1996). This may be due to a failure of NK cells to traffic efficiently to solid tumors, decreased expression of activating ligands by solid organ tumors, and/or other mechanisms that may inhibit NK cell function. The importance of tumor location is documented by the observation that Ly49G2 blockade using $F(ab')_2$ of the 4D11 monoclonal antibody enhanced rejection of the H-$2^k$ T cell lymphoma line B2-Sp3 when transferred intravenously into AKR recipient mice ($Ly49G2^{AKR}$ is identical to $Ly49G2^{BALB}$ and ligates H-$2D^k$), whereas the same treatment did not enhance rejection when the same tumor was implanted subcutaneously into the flank (Barber et al. 2008). With respect to solid tumors, combining high-dose IL-2 therapy with blocking Ly49C and Ly49I by using $F(ab')_2$ fragments limited the growth of B16-F10 (H-$2^b$) melanomas, whereas either therapy alone had minimal effect on tumor growth (Vahlne et al. 2010). Despite resulting in tumor elimination, this combination therapy did not break tolerance to normal self, suggesting that either normal self inhibits NK cells by receptors other than Ly49C and Ly49I or that B16-F10 expresses activating ligands not found on normal cells. Also, long-term blocking of Ly49C and Ly49I with 5E6 $F(ab')_2$ did not render these NK cells "unlicensed" or "disarmed" (Vahlne et al. 2010). Collectively, these studies demonstrate that blocking the inhibitory receptors for MHC class I enable NK cells to attack and eliminate both hematopoietic and solid tumors, thereby providing a new therapeutic strategy for cancer immunotherapy. One such blocking human monoclonal antibody (1-7F9) reactive with several inhibitory human KIRs increased NK cell-mediated clearance of MHC class I-expressing leukemia in humanized mice and is currently in phase I clinical trials for cancer therapy (Romagne et al. 2009).

## 7 Concluding Remarks

The Ly49 family of inhibitory receptors plays a critical role in controlling the immune functions of NK cells, first by shaping the educational and tolerant state of NK cells in the steady-state and then by augmenting or inhibiting NK cell responses to both pathogens and tumors. Many outstanding questions remain. First, we need a fuller understanding of the MHC class I ligand repertoire and affinities for the Ly49 receptors in haplotypes other than C57BL/6. Second, the molecular mechanism controlling education via Ly49 receptors remains an unanswered question. Whether developing NK cells gain responsive capacity only after expressing a self-reactive inhibitory receptor (licensing or arming) or alternatively, whether NK cells are innately responsive and hyporesponsiveness is a result of chronic stimulation unchecked by inhibitory receptors (disarming) is presently unresolved. Moreover, studies are needed to address the in vivo significance of viral down-regulation of MHC class I on NK cell responses in vivo (the "missing self" or "reduced self" hypothesis), as well as to determine whether the Ly49 receptors are involved in immunity to viruses other than MCMV. Finally, the clinical use of induced missing self through lack of inhibitory ligands to treat leukemia or blockade of inhibitory receptors to treat both tumors and infections is an attractive application that requires further consideration.

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