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INFLUENCE OF POLYMORPHISM ON LY-49 RECEPTOR SPECIFICITY AND FUNCTION

by

ELIZABETH TAKEKO SILVER 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY**

in

IMMUNOLOGY

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Was mich nicht umbringt, macht mich stärker.

Friederich Nietzsche

Every time you meet a situation, though you think at the moment it is an impossibility and you go through the tortures of the damned, once you have met it and lived through it you find that forever after you are freer than you were before.

Eleanor Roosevelt

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **INFLUENCE OF POLYMORPHISM ON LY-49 RECEPTOR SPECIFICITY AND FUNCTION** submitted by **ELIZABETH TAKEKO SILVER** in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in **IMMUNOLOGY**.

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ABSTRACT

The diversity and ligand specificity of activating Ly-49 receptors expressed by murine NK cells is largely unknown. A new Ly-49 activating receptor related to Ly-49A in its extracellular domain, designated Ly-49P, was recently cloned from 129 strain mice. We independently cloned an apparent allele of Ly-49P expressed by NOD and NOR mouse strain NK cells. Rat RNK-16 cells transfected with Ly-49P mediated reverse antibody dependent cellular cytotoxicity of FcR positive target cells, indicating that Ly-49P can activate NK-mediated lysis. We determined that RNK-16 lysis of Con A blasts induced by Ly-49P was MHC dependent, resulting in efficient lysis of H-2D^d bearing targets. We have also cloned a new Ly-49 activating receptor, expressed by NK cells of the NOD mouse strain, that we have designated Ly-49W. Ly-49W is highly related to the known inhibitory receptor Ly-49G in its carbohydrate recognition domain. Ly-49W, like Ly-49P, can activate NK-mediated lysis. We further show that Ly-49W is allo-MHC specific: Ly-49W transfectants of RNK-16 only lysed Con A blasts expressing H-2^k or H-2^d haplotypes and antibody blocking experiments indicated that H-2D^k and D^d are ligands for Ly-49W. Ly-49W is the first activating Ly-49 receptor demonstrated to recognize an H-2^k class I product. Both Ly-49P/Ly-49A and Ly-49G/Ly-49W represent new pairs of NK receptors with very similar ligand binding domains, but opposite signaling functions.

A number of Ly-49 genes exhibit allelic variation, but the functional significance of allelic differences in extracellular domains of Ly-49 receptors regarding ligand specificity is largely unknown. Amino acid differences exist in the extracellular domains of the B6 and BALB/c allele products of the inhibitory Ly-49G receptor. We found that

the ectodomain of the BALB/c allele of Ly-49G recognizes both H-2D^d and D^k class I MHC alleles, whereas the ectodomain of the B6 allele of Ly-49G only recognizes D^d, and not D^k. Furthermore, the ectodomain of the Ly-49G$^{BALB/c}$ allele recognizes a distinct repertoire of xenogeneic ligands that only partially overlaps with that recognized by Ly-49G^{B6}. Our results indicate that allelic variation in Ly-49 extracellular domains can have functional significance by altering Ly-49 receptor specificity for mouse class I MHC and xenogeneic ligands.

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LIST OF ABBREVIATIONS

Ab	antibody
ADCC	antibody dependent cell cytotoxicity
APC	antigen presenting cell
ATCC	American Type Culture Collection
β_2m	beta$_2$ microglobulin
B6	C57BL/6
B10	C57BL/10
cDNA	complementary DNA
Con A	Concanavalin A
^{51}Cr	$Na^{51}CrO_4$
CRD	carbohydrate recognition domain
CTL	cytotoxic T lymphocyte
E:T ratio	effector to target cell ratio
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GVHD	graft-versus-host disease
GVL	graft-versus-leukemia
HLA	human leukocyte antigen
HR	hybrid resistance
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
KIR	natural killer immunoglobulin-like receptor
LAK	lymphokine-activated killer cells
LRC	leukocyte receptor complex
mAb	monoclonal antibody
MCMV	murine cytomegalovirus
2-ME	2-mercaptoethanol
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid

NK cell	natural killer cell
NKC	natural killer gene complex
NOD	non-obese diabetic
NOR	non-obese diabetes resistant
NWNA	nylon wool nonadherent
PA	soluble protein A
PA/PG	mixture of soluble protein A and protein G
PBS	phosphate buffered saline
Rae1 protein	retinoic acid early-1 protein
rADCC	reverse antibody dependent cellular cytotoxicity
SD	standard deviation
SH2	Src Homology 2 domain
SHIP	SH2-containing 5' inositol phosphatase
SHP-1	SH2 domain-containing protein tyrosine kinase-1
TAP	transporters associated with antigen processing
TCR	T cell receptor
ULBP	UL16 binding protein

CHAPTER I

INTRODUCTION

A. Function and Properties of NK Cells

Natural killer (NK) cells were first described in the 1970s, but only recently have many important aspects of their biology been elucidated.

In the early 1960s, cytotoxicity directed against transplantated tissue was found to be regulated by an adaptive immune response of certain lymphocytes against antigens encoded by the major histocompatibility complex (MHC) (1, 2). This specific adaptive response was found to be mediated by cytotoxic T lymphocytes (CTLs) because of their dependence on the thymus for maturation (3). Later, it was determined by Zinkernagel and Doherty (studies for which the 1996 Nobel Prize was awarded) that viral antigens on target cells were recognized only when they were associated with the target cell MHC proteins (4). In contrast to this CTL-mediated cytotoxicity which required prior exposure to the pathogen presented to the T cell, several publications during the period of 1975 to 1977 noted that some lymphocytes, lacking both T and B cell markers, could spontaneously kill target cells without prior exposure or known sensitization (1). This spontaneous cytolytic activity was called "natural cytotoxicity" and these cytotoxic lymphocytes, which in contrast to CTL appeared to lack MHC restriction, were defined as "natural killer" (NK) cells. NK cells do not require rearrangement of receptor genes as do T cells and B cells. When either of the recombinase genes *RAG-1* or *RAG-2* are disrupted, mice are unable to produce either T cells or B cells, but NK cells develop

normally and are capable of mediating natural cytotoxicity (5, 6). There is also a population of $CD3^+$ T cells that mediate natural cytotoxicity, become depleted with the anti-asialo-GM_1 antiserum treatment that was commonly used to eliminate NK cells, express several classical NK cell markers, and have been named "natural T cells" or NKT cells. The NKT cells have a limited T cell receptor (TCR) repertoire and rely on the nonclassical MHC class I molecule CD1 for their development and recognition of glycolipid antigens (7, 8). Because of their similarity to NK cells with respect to cellular markers, NKT cells were not distinguished from NK cells in many of the early studies that defined the function of NK cells.

NK cells play a role in early defense against some viruses, especially the herpesviruses. Human patients with a complete absence of NK cells have been rare, but the few cases reported have shown recurrent and severe viral infections, including those by herpes simplex virus, Epstein-Barr (EBV), varicella-zoster virus, papillomavirus, and cytomegalovirus (1, 9). In mice, depletion of NK cells results in increased sensitivity to several viruses including murine cytomegalovirus (MCMV), herpes simplex, influenza, and Coxsackie viruses (10).

Many early studies on the NK cell killing of tumor cells used beige mice, the mouse model of Chediak-Higashi syndrome, which features a deficiency of NK cell function (11), but the beige mouse is not totally appropriate as a model for NK deficiency. These mice have defective NK cytotoxicity due to a defect which affects the granules of not only NK cells, but many other cytotoxic cells as well, and the beige mutation also does not diminish other NK cell activities such as cytokine production (12, 13). NK cells are involved in the control of tumors, but several studies have shown that

NK-like cytotoxicity of tumor cells can be the result of effector cells that are actually NKT cells and not NK cells (14, 15). It was subsequently determined that NKT cell activation rapidly results in the activation and proliferation of NK cells, and the production of IFNγ by NK cells (16, 17). More recently, an NK cell-deficient mouse has been described, and this mouse exhibited a failure to reject tumor cells *in vivo*, even though NKT cells are present in this mouse (18), suggesting that NK cells themselves are important in suppressing tumor metastasis. Recently, a feedback loop has been described where dendritic cells (DCs) mediate NK activation, and activated NK cells in turn activate naïve DCs, except in the case of an overwhelming NK cell response when DCs are killed by the activated NK cells, presumably to limit the inflammatory response (19-21). Thus, while sometimes considered simple, nonspecific and primitive, the NK cell is actually a significant participant in the integration of innate and acquired immune responses.

B. NK Cells and Receptor-Mediated Inhibition

NK cells were found to play a pivotal role in two phenomena that puzzled researchers for several years after the discovery of NK cells. The confirmation that CTL expressed a receptor that recognized a specific ligand on a target cell, along with the observation of the similarities between cytotoxic T cells and NK cells, gave rise to the idea that NK cells expressed a unique NK receptor that could recognize a specific ligand present on NK-sensitive target cells, such as virally infected or tumorogenic cells (22). The search for the unique NK cell receptor was hindered by the existence of two enigmatic NK cell phenomena: (1) an inverse correlation between class I MHC

expression on target cells and their sensitivity to NK-mediated cytolysis (23), and (2) rejection of parental bone marrow grafts by their F1 hybrid offspring, a phenomenon called "hybrid resistance" in bone marrow transplantation (24).

The "Missing Self" Hypothesis: NK Cell Regulation by Target Cell MHC

To account for the increased NK cytotoxicity of class I-deficient tumor cells, Klaus Kärre proposed his "missing self" hypothesis (Fig. 1-1) in which one role of NK cells was to identify pathogenic cells that had lost class I expression and would thus be overlooked by T cells (23). He suggested two alternative mechanisms by which NK cells could sense "missing self": the "target interference" model in which class I proteins somehow sterically interfered with the binding of NK activation receptors to their target-cell ligands, and the "effector inhibition" model where class I proteins on a potential target cell delivered a 'negative signal' to inhibit NK cell cytotoxicity. The discovery of the inhibitory receptor Ly-49 (Ly-49A) on mouse NK cells (25) that could bind to purified class I molecules (26) corroborated the "effector inhibition" model and gave additional support to the "missing self" hypothesis. The human NK cell surface molecule p58 (KIR2DL) was also determined to be an inhibitory receptor (27), establishing the paradigm also held for human NK cells.

Hybrid Resistance

In the phenomenon called "hybrid resistance" (HR), F_1 hybrid mice reject bone marrow cells from either homozygous parent P_1 or P_2, while the F_1 bone marrow cells are accepted by both parents. This contradicts the classic laws of transplantation

immunology, where tissue graphs from homozygous mice are accepted by their F1 offspring while the parental mice reject tissue from F1 offspring. Two theories were consistent with HR. One theory was that HR was due to the existence of hemopoietic histocompatibility (Hh) antigens that had a recessive pattern of inheritance (28). The other theory stated that NK cells recognize and eliminate cells lacking self-class I MHC that would cause NK cell inhibition only for cells expressing self-class I MHC (the "missing self" hypothesis). Since some F_1 NK cells would identify bone marrow cells from P_2 as missing some self class I molecules, that subset of NK cells would kill those P_2 bone marrow cells (29). When no Hh antigens could be uncovered, and with the conclusion that HR-like target killing and parental bone marrow graft rejection could be induced by the interruption of the binding of NK inhibitory receptors to their class I ligands, the "missing self" hypothesis came to be accepted as an explanation for hybrid resistance (30, 31).

C. Class I MHC

A fundamental characteristic of immune systems is the ability to distinguish "self" from "non-self". Rejection of foreign tissue results from recognition and immune response to cell surface antigens that were identified, characterized, and named histocompatibility antigens by Peter Gorer and George Snell. This work done in 1930s to 1950s resulted in a Nobel Prize in 1980 for Snell (with Jean Dausset and Baruj Benacerraf) (32) after Gorer's death. The ability of mice to reject foreign tissue was found to be mediated by the recognition and immune response of cytotoxic T cells to major histocompatibility antigens mapped to the H-2 (for "histocompatibility-2") region

on chromosome 17 which became known as the H-2 complex, or major histocompatibility complex (MHC) (33). The human MHC, called the Human Leukocyte Antigen (HLA) complex, was mapped to chromosome 6 (34). The rat MHC is known as the RT1 complex and is located on rat chromosome 20 (35). Although MHC regions are found in all vertebrate species from fish to mammals, most of the research has been done on the mouse and human MHC, with considerable work also done on the rat MHC (36, 37). The MHC regions for all three species are organized into three classes of genes: class I gene products are expressed on most cell types, class II gene products are expressed mainly on antigen presenting cells (APCs), and class III gene products are a diverse set of proteins, many of which have immune function, such as components of the complement pathway and tumor necrosis factors TNF-α and -β.

Class I MHC proteins are composed of two non-covalently associated polypeptides: the glycosylated, 44 to 47 kDa heavy (or α) chain and the 12kDa beta$_2$ microglobulin (β_2m) light chain (Fig. 1-2A). The β_2m protein is highly conserved among species, and the β_2m from one species can often associate with a heavy chain from another species. Association of β_2m is necessary for the expression of stable class I proteins, and class I α chains are not expressed on the cell surface in cells deficient in β_2m, such as the human Daudi cell line (38). The α chain folds into three extracellular domains, α1, α2, and α3, on a flexible stalk with a single transmembrane segment spanning the cell membrane and a cytoplasmic domain. The α3 domain interacts with β_2m, while the α1 and α2 domains form a peptide binding cleft from four β strands contributed by each of the α1 and α2 domains that interact to compose a platform upon which two α helices (one from each of the two domains) act as the walls of the cleft (Fig.

1-2B). T cells interact with class I when the TCR binds to residues on the tops of the two α helices of the peptide binding groove and the bound peptide. The protein binding groove binds short self peptides, usually 8 to 10 residues long, derived from the proteolytic digestion of endogenous proteins by the proteasome in the cytosol (39). The peptides are then transported by the transporters associated with antigen processing (TAP) transporter complex into the endoplasmic reticulum, where the peptides interact with the newly-synthesized class I and β_2m (40). The class I- β_2m-peptide complex is transported to the cell surface via the Golgi apparatus. The class I proteins are not stable in the absence of high affinity peptide, and cells deficient in the TAP transporter express class I that is unstable at 37°C but can become stable with exposure to exogenously added high affinity peptide (41-43). While these unstable class I proteins were previously termed "empty" class I molecules (41), they were later renamed "peptide-receptive" class I because of the possibility that the peptide binding groove had bound a low affinity peptide that was easily displaced, as opposed to no peptide at all (44, 45).

Class I MHC proteins are classified into two groups, class Ia (also called "classical" class I) and class Ib (also called "non-classical" class I). The class Ia MHC proteins are expressed on the surface of most nucleated cells. Class Ia heavy chains are encoded in gene regions named K, D, and L in mouse, A, B, and C in humans, and RT1.A in rats. The number of class I genes can vary in number between different strains of mice and rats, while most humans express three class I genes. In the mouse, H-2K and H-2D are present in all strains, while H-2L is absent in some mouse strains such as C57BL/6. Different rat strains have been found to express one, two or three class Ia genes (36). Class Ia MHC genes have a highly conserved α3 domain, while the α1 and

α2 domains exhibit extreme polymorphism. This pattern is consistent with the ability of the peptide binding groove of class Ia MHC to present a widely diverse range of peptides to the TCR. In contrast to class Ia MHC, class Ib genes are expressed in a limited number of cell types, exhibit limited polymorphism, and an antigen-presentation function has been shown for only some of the class Ib proteins, which appear to present peptides or macromolecules of limited variability (37, 46). The mouse and human class Ib genes are encoded in the MHC (46). CD1 is a class I-like molecule that is not MHC-encoded (46). Rat class Ib genes are located in the RT1-C/E/M regions of the MHC. There are estimated to be more than 50 class Ib genes in both the mouse (47) and rat (37, 48), while estimates of human class Ib genes is smaller (49).

Class I-like genes that are structurally related to class I genes include several proteins frequently expressed on tumors but not on normal tissue: in mouse, the retinoic acid early-1 (Rae1) family of proteins Rae1α-δ, and the H-60 minor histocompatibility antigen; in humans, the UL16 binding proteins ULBP-1, 2, and 3 (a family of proteins that binds to the CMV protein UL16), and the stress-inducible proteins MICA and MICB. These class I-like proteins have α1 and α2 domains homologous to class I, but do not associate with β_2m and do not bind peptide (50, 51).

D. The KIR and the Ly-49 Gene Clusters

Inhibitory or activating NK receptors in both humans and mice belong to one of two structurally dissimilar groups, the immunoglobulin (Ig) or the C-type lectin superfamilies. Most of the genes for these receptors are encoded in two clusters, with the

C-type lectins encoded in the natural killer gene complex (NKC) and the Ig receptors encoded in the leukocyte receptor gene complex (LRC).

Human NK cell cytolytic activity is modulated by the natural killer Ig-like receptors (KIRs). *KIR* genes are not found in the mouse genome, and the only Ig-superfamily inhibitory receptor expressed in mice is the unrelated, activation-induced gp49 (52). The human *KIR* gene family is encoded in the LRC in the *KIR* gene complex on human chromosome 19. The *KIR* genes are arranged in tandem with the same transcription orientation, and the *KIR* complex is approximately 150 kb in size and contains no other type of genes. (The human LRC also contains the loci for other receptor gene families, like the KIR-related immunoglobulin-like transcript (*ILT*) genes (53) and the leukocyte-associated Ig-like receptor (*LAIR*) genes (54), and some may be expressed on NK cells, but these receptors generally appear to participate in the function of B, T, and/or myeloid cells.) Over 100 closely related human *KIR* sequences are listed in GenBank, but KIR proteins have been classified into 13 groups based on number of Ig domains (2 or 3), long or short cytoplasmic tail (L or S), and sequence similarity: KIR3DL1-2, KIR3DS1, KIR2DL1-5, and KIR2DS1-5. KIR proteins can have cytoplasmic tails of different lengths, and those with long cytoplasmic tails (except for KIR2DL4) contain a pair of the sequences named the immunoreceptor tyrosine-based inhibitory motif (ITIM), which recruits and activates the SH2-containing protein tyrosine phosphatase SHP-1 (55), and these KIR are inhibitory receptors; conversely, KIR with short tails have no ITIMs due to premature stop codons, and are activating receptors. The activating KIR receptors also possess a positively charged residue in their transmembrane domains which allows interaction with the immunoreceptor tyrosine-based activation

motif (ITAM)-containing signaling molecule DAP12, also called killer cell activating receptor-associated protein (KARAP). Comparison of *KIR* genes in different individuals has revealed that *KIR* genes can exhibit gene duplication, as well as gene recombination with other *KIR* genes or with a pseudogene fragment resulting in pairs of genes whose products have similar ligand binding capabilities but differ in their possession of ITIMs. Some examples of gene pairs thus formed include 2DL1/2DS1, 2DL2/2DS2, and 3DL1/3DS1. It should be mentioned that the *KIR* gene family is plastic and rapidly evolving (56), complicating the differentiation between genetic alleles and distinct (but similar) genetic loci. The convention that has been followed is that similar sequences that possess more than 20 nucleotides differences are probably different genes, while those that differ by less than nine nucleotides are likely alleles. There are at least a dozen KIR genes but the number varies between individuals, as the analysis of just two individuals showed they differed in their number of KIR genes (57).

The non-Ig inhibitory receptors are type II, integral membrane proteins of the C-type lectin superfamily. Several C-type lectin receptors are important in the inhibition or activation of NK cells including CD94, natural killer group protein 2 (NKG2), natural killer cell receptor P1 (NKR-P1), and Ly-49. The lectin receptor genes are encoded in the NKC, located on chromosome 12 in humans and on chromosome 6 in the mouse. CD94 forms heterodimers with NKG2A, C, and E (58). The inhibitory CD94/NKG2A and the activating CD94/NKG2C and NKG2D receptors are expressed in human, mouse and rat genomes, while only one functional *NKR-P1* receptor gene (*NKR-P1A*) and one nonfunctional *Ly-49* pseudogene (*Ly-49L*) are encoded in humans (59). Baboons express both functional Ly-49L transcripts and multiple KIR genes, suggesting that the loss of

functional Ly-49 in humans occurred 6-10 million years ago (60). The mouse NKR-P1 family includes the activating receptors NKR-P1A and NKR-P1C (also frequently called NK1.1), an inhibitory receptor NKR-P1B (61), and the genes for NKR-P1D and F were recently reported (62).

The *Ly-49* gene family, like the *KIR* gene family, is composed of many different, closely related members. Chronologically named, cDNA sequences for Ly-49 receptors from A to W are currently listed in GenBank, although *ly49k* and *n* have only been found as gene fragments. All the *Ly-49* genes, except for *Ly-49B*, are in a cluster in a region thought to be ~420 kb in length (63). Like the *KIR* gene cluster, the *Ly-49* gene cluster has genes arranged in tandem with the same transcription orientation, the genes appear to be rapidly evolving, different sequences cannot be confidently identified as different genes or different gene alleles, and the analysis of the C57BL/6 and 129/J *Ly-49* gene clusters demonstrates that the number of genes differs between inbred mouse strains (64, 65).

Recently, inbred mouse strains were classified based on the available published information on strain derivations and inbreeding histories to create a record of inbred mouse genealogies (66). Subsequently, twenty-two different inbred mouse strains from four of the seven inbred mouse lineage categories were analyzed with 26 sequence tagged site (STS) *Nkc* markers derived from the C57BL/6 mouse strain to determine *Nkc* locus allotypes (67). The authors found that *Nkc* allotypes generally fell into patterns consistent with their genealogical classification. Analysis of the *Ly-49* gene clusters from different putative *Nkc* allotypes may help to clarify the uncertainty in differentiating gene

loci from gene alleles, but the delineation and description of *KIR* locus haplotypes *A* and *B* in the human KIR system has not greatly helped to resolve this issue (56).

E. NK Activating Receptors for Non-MHC ligands

The search for the activating receptors involved in "natural cytotoxicity" (the ability of NK cells to kill antibody-free target cells without prior sensitization) has resulted in the identification of several NK receptors. The low affinity IgG receptor CD16 (FcγRIII) is the most extensively characterized of the activating receptors (68). CD16 binds the carboxy terminal (or F_c) region of IgG antibody molecules, allowing NK cells to recognize IgG-coated cells and kill these targets by a process termed antibody dependent cell cytotoxicity (ADCC). Signaling is mediated through the noncovalent association of FcγRIII with dimers of the ITAM-bearing adaptor protein of FcεRIγ in mice, or either FcεRIγ or CD3ξ in humans. Cross-linking of CD16 by ligand binding or antibody results in NK degranulation and IFNγ production by a signaling pathway involving the tyrosine phosphorylation of ITAMs by $p56^{lck}$. CD16 has not been found to be involved in NK cytotoxicity in the absence of antibody. In mice, the protein NKR-P1C (also named NK1.1) of the NKR-P gene family activates NK degranulation and IFNγ production upon binding to an unidentified ligand on target cells or upon antibody cross-linking (69). NKR-P1C is also expressed by NKT cells and it can be expressed by $CD8^+$ T cells (70). As well, the NKR-P genes are not expressed in all mouse strains; i.e. BALB/c does not express NKR-P1C and has NK cells that apparently function normally. Three NK-specific receptors have been cloned that are involved in the activation of NK cells during the process of natural cytotoxicity. These three human natural cytotoxicity

receptors (NCR) NKp46 (71), NKp30 (72), and NKp44 (73), appear to be unrelated to other molecules of the immunoglobulin (Ig) superfamily or each other. Of these NCR, only NKp46 is found in the mouse (74). The ligands for these receptors have not yet been determined, but antibody blocking of these receptors resulted in strong inhibition of cytolysis. Another potential receptor for natural cytotoxicity is NKG2D. It is expressed on NK cells, activated $CD8^+$ T cells and activated macrophages in mice, and in all NK cells, $CD8^+$ and γδ T cells in humans (50). Its ligands have been determined to be class I-like molecules (described briefly above): the stress-inducible MICA, MICB, and ULBP proteins in humans, and the RaeI proteins and H60 (a minor histocompatibility antigen) in mice (75-77). The human ligands for NKG2D are expressed predominantly (but not exclusively) by cells of epithelial origin (78), while the mouse NKG2D ligands are normally not expressed by any cells from adult mice (50). Since *in vivo* NK cells appear to predominantly target hematopoietic cells as opposed to epithelial cells (79, 80), it is likely that the NKG2D receptor is not a major player in natural cytotoxicity, although it may still operate in the recognition of target cells by NK cells. The known NKG2D ligands appear to be up-regulated in stressed cells, such as tumor cells or virally-infected cells (50), and NKG2D may be involved in the activation of cytolytic lymphocytes other than NK cells (50, 81).

F. NK Receptors for Class I MHC

The CTL-mediated clearance of transformed or virally infected cells requires that the CTL can sense pathogenic peptides bound to self class I. Some tumors and several viruses have developed many different mechanisms for down-regulating the cell surface

expression of class I (82, 83). Likewise, NK cells have developed several strategies for recognizing missing self class I. NK inhibitory receptors can recognize class I either directly or indirectly. The direct binding of specific class I ligands is accomplished by KIR in humans and Ly-49 in mice. The indirect detection of class I-expressing cells is carried out by heterodimers of CD94 and NKG2 proteins which recognize leader sequence peptides presented by the non-classical class I proteins HLA-E in humans (84) and Qa-1B in mice (85). CD94 is invariant and is encoded by a single gene, while NKG2A, C, and E are homologous members of a multigene family. (NKG2D, which has been discussed in an earlier section, shows little homology to the other NKG2 members, recognizes different ligands, and does not associate with CD94.) NKG2A is an inhibitory ITIM-bearing receptor, while NKG2C and E are activating receptors that associate with DAP12.

Although the KIR and Ly-49 receptors are very different structurally, they use similar signaling pathways to mediate NK inhibition (Fig. 1-3). The cytoplasmic tails contain the ITIM consensus sequence (I/V)xYxx(L/V) (55). Binding of the inhibitory receptor to class I ligand on the target cell causes aggregation of the inhibitory receptors to the cell-cell contact zone (86) where the activation receptors, such as the NCR, also aggregate, resulting in tyrosine phosphorylation in the ITIM (87). It is probable that proximity of the inhibitory receptors to the activating molecules causes ITIM phosphorylation by the Src family kinase such as $p56^{lck}$ used in the activating pathway (88). The phosphorylated ITIM allows the recruitment of the Src homology2 (SH2) domain-containing protein phosphatase SHP-1 (55, 89). SHP-1 is presumed to dephosphorylate substrates critical to the cellular activation pathway, although a crucial

SHP-1 target has not been determined (90). Another mechanism that could work in coordination with (or result from) substrate dephosphorylation is disruption of the lipid raft redistribution to the cell-cell contact point that is usually seen in human NK cells upon activation but that does not occur upon KIR engagement (91).

Regulation of immune responses by the coordinated actions of activation and inhibition is a common theme in immunology (92). The NK inhibitory receptor families have all contained non-inhibitory family members (93). They all have in common the existence of a charged residue in the transmembrane domain and removal of the ITIM, by truncation for most of Ig-family receptors and by replacement of tyrosine by phenylalanine for the lectin-type family members. In the case of KIR and Ly-49, activators have a positively charged residue in the transmembrane region, lysine for KIR (except the unusual activator KIR2L*A*, which has arginine) and arginine for Ly-49 (Fig. 1-3). This positively charged residue interacts with the ITAM-bearing adaptor protein DAP12 by an ionic interaction with their transmembrane domains (94).

The *Ly-49* gene clusters of the C57BL/6 and 129/J mouse strains have been examined in the most detail (64, 65, 95). The C57BL/6 mouse expresses Ly-49A to J, and four pseudogene fragments *ly49k* to *n*. Six activating Ly-49 genes have been found in these mouse genomes, but complete mRNA transcripts are found for only two in the C57BL/6 mouse (*Ly-49D* and *Ly-49H*) and three in the 129/J mouse (*Ly-49P*, *Ly-49R* and *Ly-49U*). Interestingly, pseudogenes have been found for several activating Ly-49 genes, while no inhibitory pseudogenes have been found in these mice. It has been suggested that activating Ly-49 genes are more rapidly evolving, perhaps to defend against new pathogen variants (65). This hypothesis is supported by the finding that mouse resistance

to the murine cytomegalovirus is dependent on the activating receptor Ly-49H (96-98) and mice with a nonfunctional version of DAP12 (and thus with nonfunctional Ly-49H) are not able to mount a good resistance against the murine cytomegalovirus (99). MCMV down-regulates the host cell surface expression of host class I MHC using multiple strategies (100). The down-regulation of class I protects against CTL recognition and cytolysis but could trigger the activation of NK cells. To counter this, the viral gene product m157 binds to inhibitory NK receptors in certain MCMV-susceptible mice, and to the activating receptor Ly-49H in the MCMV-resistant B6 mouse strain (101).

The activating molecule Ly-49D has recently been found to mediate the cytolysis of hamster and rat cells. Chinese hamster ovary (CHO) cells are lysed by C57BL/6 NK cells, but not by BALB/c NK cells, and this recognition is mediated by Ly-49D (102, 103). Recently, the cloning of five classical class I cDNAs from CHO cells has been reported and one of these class I proteins, Hm1-C4, has been demonstrated to bind to Ly-49D (104).

G. Regulation of Ly-49 Expression

Fetal mouse NK cells are generally deficient in Ly-49 expression, with Ly-49A, B, C, D, F, G, H and I undetectable by antibody staining of cell lines generated from 14-day fetal cells, or by bulk RT-PCR of 17-day fetal NK cells (105, 106). The only Ly-49 receptor expressed by fetal NK cells appears to be Ly-49E (106, 107), which is expressed at levels 10 to 30 times higher than seen in uncultured NK cells from adult mice, although IL-2-activated NK cells from adult mice up-regulate their Ly-49E expression to

that of fetal NK cells (106). Sixty four NK cells from one week old neonatal mice analyzed by single cell RT-PCR expressed very low levels of Ly-49A, C, H and I transcripts, but not Ly-49E (nor Ly-49B, D, F, G, or J) transcripts (108). The same study also analyzed 62 NK cells from eight week old mice and showed that over 30% expressed transcripts for Ly-49A, C, D, G, G, and I, while 15% expressed Ly-49H and 5% expressed Ly-49J, and none expressed Ly-49B, E, or F transcripts. It thus appears as if the expression of Ly-49E is high in fetal NK cells, but drops sharply within a week of birth and remains low in the absence of NK cell activation.

Adult NK cells appear to be able to express transcripts from one to six Ly-49 receptor genes (108). The distribution of Ly-49 receptors in a population of NK cells appears to be random, with each Ly-49 receptor being distributed in an independent, or stochastic, manner (109). An independent distribution of Ly-49 receptors would allow an estimation of the frequency of NK cells expressing a given combination of receptors by multiplying the frequency of NK expression of each receptor (the "product rule"). Examples of deviation from the product rule indicate factors other than stochastic distribution can affect Ly-49 expression. For example, activating Ly-49 receptors may be regulated by a different process than inhibitory receptors, as C57BL/6 NK cells that express the activating receptor Ly-49H have a tendency to coexpress the other activating B6 receptor Ly-49D at a frequency greater than estimated by the product rule (110). Also, heterogeneous B6xBALB/c mice encode the genes for two alleles of Ly-49A and Ly-49G; while their expression is predominantly monoallelic on individual NK cells, the numbers of NK cells expressing both alleles is greater than that expected from the product rule (111).

The level of Ly-49 expression is regulated by class I. The "receptor calibration" model suggests that the level of class I in the environment of the NK cell regulates the expression of their inhibitory Ly-49 receptors on NK cells so that reduced levels of class I molecules can be detected (112). Another model states that down-modulation of Ly-49 receptors is dependent on their frequency of interaction with their class I ligands, so that similar environments could generate either many Ly-49$^+$ NK cells with low expression levels or few Ly-49$^+$ NK cells with high expression levels (113). The levels of Ly-49 expression are not fixed, as mature Ly-49A$^+$ NK cells introduced into a different mouse modulate their Ly-49A levels depending on their new class I environment (114, 115).

The down-regulation of Ly-49 receptors that recognize self class I may be explained by the recent finding that the binding of Ly-49 or KIR to class I results in the transfer of class I ligand from target cell to the NK cell and receptor internalization (116-118). The inhibitory receptors Ly-49A and Ly-49G mediate the transfer of target cell D^d to the NK cell and became internalized, but D^d transfer and internalization has not been seen for Ly-49D, leading to the suggestion that the ITIM somehow helps mediate the class I transfer so that only inhibitory receptors transfer ligand (116). Ly-49A mediates the transfer and uptake only of its strong ligands D^d and D^k, and not of its weak ligand D^b (117), thus the internalization of Ly-49 receptors that bind self ligand strongly may be a mechanism to decrease the surface expression of self-reactive Ly-49 receptors. The transfer of antigen from target cells to lymphocytes may be a recurrent mechanism to regulate cellular lymphocyte function since it has been demonstrated with B cells and has been suggested to play a role in its ability to present antigen to T cells (119), and T cells have also shown the TCR-mediated transfer of class I and class II (120-123).

There is evidence that the SH_2-containing 5' inositol phosphatase SHIP is involved in the down-regulation of inhibitory Ly-49 receptors that recognize self class I (124). SHIP appears to be important in the selection of NK cell subsets expressing different Ly-49 inhibitory receptors, possibly by limiting the *in vivo* survival of some NK subsets so that the number of Ly-49 inhibitory receptors expressed by each cell is limited. In SHIP deficient B6 cells, the self-specific receptors Ly-49A and Ly-49C (but not Ly-49G2, Ly-49F, or Ly-49I) are co-expressed on the majority of NK cells, and these mice are unable to reject fully mismatched allogeneic bone marrow grafts (124). This suggests that SHIP operates to limit the expression of self-reactive NK inhibitory receptors to no more than one per cell so that the NK cell population will be able to kill target cells that are "missing self".

$CD8^+$ TCRαβ NK T and T cells can acquire expression of NK1.1, Ly-49A, Ly-49C/I and Ly-49G, but not Ly-49D, after exposure of cytokines IL-2, IL-4 or IL-15 (70). Since the receptors of these cytokines use the common γ chain, a pathway involving the common chain seems to be operating for up-regulation of inhibitory receptors for class I on $CD8^+$ T cells. *In vivo* virus infection also up-regulates NKR-P1C and Ly-49A, C/I, G, and unusually, a small amount of Ly-49D (125). CTL expressing inhibitory Ly-49 also appear to carry markers of memory T cells, indicating that inhibitory receptor expression may be induced by prior or chronic activation (126). The inhibition by Ly-49 is likely not a simple "off" switch, but Ly-49A expressed by T cells was shown to modulate cytolytic responses so that higher levels of stimulus are required to reactivate the T cell (127, 128). Inhibitory Ly-49 receptors may play a similar role on NK cells.

H. Binding of Ly-49 to ligands

The ability of NK cells to contact multiple potential targets simultaneously has been seen with human (118) and mouse (129) NK cells. When a target expressing the ligand for an inhibitory receptor on the NK cell, the NK cell cytolysis of that resistant cell is inhibited while susceptible target cells simultaneously bound are killed (there is no "bystander" killing or inhibition) (129). Also, it appears that resistant targets interact with the NK cell for shorter time periods than do susceptible targets (129).

The B6 allele of Ly-49A binds class I molecules H-2D^d and D^k (25, 26, 130, 131) and H-2D^b tetramers with low affinity (132). COS cell transfectants of the B6 allele of Ly-49A bound Con A blasts from H-$2^{d, k, f, q, r, s, v}$ mice (131). COS cell transfectants of both B6 and BALB/c alleles of Ly-49C have been found to bind to H-$2^{d,b,k,s}$ cell lines (133) and to Con A blasts from H-$2^{b, d, k, f, q, r, s, v}$ mice and to tetramers of H-D^b, K^d, D^d, and D^k (131). Ly-49C was also found to have low affinity binding to H-2K^b (131, 132). Compared to the B6 alleles of the other inhibitory Ly-49 receptors, the B6 alleles of Ly-49A and Ly-49C appear to bind a wider range of class I ligands with a high affinity. However, these two inhibitory receptors appear to differ in the role played by the peptide bound by the class I. Ly-49A recognizes D^d independently of bound peptide sequence, but does require peptide as it does not recognize "peptide-receptive" D^d (134, 135). In contrast, Ly-49C inhibition of NK cell killing of RMA-S cells expressing K^b either varied according to the peptide used to stabilize K^b (136) or diminished in proportion to the level of high-affinity peptide bound to the "peptide receptive" K^b (43). In either case, Ly-49C recognition of class I appears to be influenced by the peptide bound.

Class I specificity has been demonstrated for some of the other Ly-49 receptors. Ly-49G has been reported to be a receptor for H-2D^d and/or L^d as antibodies for these class I molecules or the Ly-49G antibody 4D11 allowed target cell cytolysis by 4D11$^+$ cells (137). The results of this experiment turned out to be ambiguous when the 4D11 antibody was demonstrated to cross-react with the D^d receptor Ly-49A (133, 138). The issue was resolved when tetramers of D^d, but not L^d, were found to bind weakly to COS cells transfected with the B6 allele of Ly-49G (131). Ly-49I has been reported to bind K^d tetramers strongly and D^d tetramers weakly in a peptide dependent manner (131). The B6 allele of the activating receptor Ly-49D recognizes D^d, but with much weaker affinity than does Ly-49A (139, 140) as well as D^r and D^{sp2} (141). Ly-49D also mediates lysis of the Chinese hamster ovary (CHO) cell line (102, 103) and targets derived from the MHC congenic rats of the RT1^{lv1} and RT1^l haplotypes (102) which share the rat classical class I RT1.A^l (142). Some characterization has been done on Ly-49 receptors from other mouse strains. Ly-49A alleles appear to interact with H-2D^d with differing avidities, suggesting that there are allelic differences in ligand interaction (143).

Ly-49A binding of D^d can be blocked by antibodies to the α1/α2 domains that define the peptide binding groove (25, 26) but Ly-49A recognition is independent of peptide (134). While Ly-49A and D^d-restricted TCR both recognize the α1/α2 domains, competition assays demonstrated that Ly-49A and TCR bind D^d at different sites (144). The crystal structure of the carbohydrate recognition domain (CRD) of Ly-49A bound to D^d shows that the Ly-49A homodimer contacts D^d on two distinct sites (145). At site 1, a Ly-49A subunit binds D^d on one side of the peptide binding platform away from the bound peptide. Site 1 is small and has a very precise match of the D^d interface surface to

Ly-49A in a region where Ly-49 receptors demonstrate great polymorphism. Its location would be consistent with the necessity of peptide occupancy in the binding groove, but because Ly-49A contact would be away from the peptide the specific peptide sequence might not matter. Site 1 has a small hollow that would fit a glycosylation at residue 176 of D^d, which would be compatible with the finding that glycosylation at residue 176 but not at residue 86 increases the avidity of Ly-49A binding to D^d, and that carbohydrate modification alters Ly-49A binding avidity (146-148). In contrast, site 2 is a large concave region beneath the peptide binding platform where both units of the Ly-49A homodimer interact with D^d, with one Ly-49A subunit contributing 79% of the Ly-49A contact surface. Site 2 overlaps with the CD8 binding area and includes interactions with β_2m, and incorporates the area on D^d with both glycosylation sites. While the angle of Ly-49A to D^d in site 2 would appear to only allow *cis* interactions (interactions between proteins on the same cell surface), the crystal structure does not include the 67 residue stalk that should allow sufficient flexibility for *trans* interactions between proteins on different cells. Site 2 does not have very good shape complimentarity between the interacting Ly-49A and D^d surfaces, and much of the area of site 2 contact is in nonpolymorphic regions of Ly-49 and class I. The residues of D^d that are polymorphic at site 2 do not explain why Ly-49A exhibits high affinity binding to D^d and not to K^b or D^b (149). Although site 1 is the more intuitive choice, mutation analyses of Ly-49A and D^d have demonstrated that some mutations in site 1 do not greatly disrupt binding of Dd, while some site 2 residues, including some in β_2m, are important in Ly-49A binding to D^d (138, 150, 151).

I. Rationale

The Ly-49 multi-gene receptor family regulates mouse NK cell functions, but the diversity and ligand specificity of Ly-49 receptors was largely unknown in June 1998 when I started the studies included in this thesis (Table 1-1). The B6 strain has served as a prototype for the study of Ly-49 gene expression and function, but my examination of the NOD mouse strain has established that it expresses a different repertoire of Ly-49 receptors from the B6 mouse. Two novel cDNA sequences expressed by the NOD mouse we named Ly-49P and Ly-49W, and they appeared to encode activating receptors with high sequence identities to the inhibitory receptors Ly-49A and Ly-49G, respectively.

The "missing self" hypothesis postulates that NK cells recognize and kill target cells that are deficient in class I MHC expression. Inhibitory class I receptors restrain NK cytolysis of potential target cells with normal class I expression, but are not engaged when the target cells express inadequate levels of class I ligands which permits the NK cell to kill those targets. The existence of class I receptors that activate NK cell cytolysis would appear to contravene the "missing self" hypothesis, since NK cells expressing these receptors might kill target cells expressing class I instead of being inhibited from killing.

Because the CRD regions of the Ly-49P and Ly-49W receptors have such a strong resemblance to their inhibitory counterparts, it seemed likely that Ly-49P and Ly-49W would recognize class I MHC ligands. Our objectives for the studies in chapters II and III were to establish, first, if the receptors Ly-49P and Ly-49W were activating receptors, and second, if they recognized class I ligands, and if so what ligand

specificities. Our rationale for doing these experiments was that the information obtained might help to clarify the role of activating Ly-49 receptors if we found, for instance, that activating and inhibitory receptor counterparts differed in either their ligand specificities or their affinity of ligand binding.

A number of *Ly-49* genes expressed in different mouse strains exhibit allelic variation, but the functional significance of allelic differences in extracellular domains of Ly-49 receptors regarding ligand specificity is largely unknown. Our studies of the ligand recognition of Ly-49P and Ly-49W with their ligands indicated that a Ly-49 sequence variable loop might be important for the specificity of ligand recognition. The B6 and BALB/c alleles of the inhibitory receptor Ly-49G exhibit sequence variation within the variable loop, suggesting that they could differ in the specificities or affinities of ligands they recognize. Demonstration of differences in ligand recognition by alleles of the same gene could give functional relevance to the observed occurrence of allelic exclusion, where only the maternal or the paternal gene is expressed in F_1 mice.

Together, these studies examine the influence of sequence variation in the ligand recognition of activating and inhibitory Ly-49 receptors.

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Table 1-1. Ligands recognized by Ly-49 receptors (published as of June 1998).

While other studies examining Ly-49 recognition of target cells from different MHC haplotypes have been described, the ligands listed in this table are from studies for which specific class I ligands were established.

	Ly-49 Allele	Ligand Recognition	Technique Used*	References for Ligand Recognition
Ly-49A	C57BL/6	H-2D^d, D^k	A, P	(25, 26)
Ly-49C	C57BL/6	H-2K^b	A	(30)
Ly-49G	C57BL/6	H-2L^d, D^d	A	(137)

* A antibody blocking of Ly-49 receptor/ligand interaction

P adhesion of Ly-49-expressing cells to immobilized purified class I

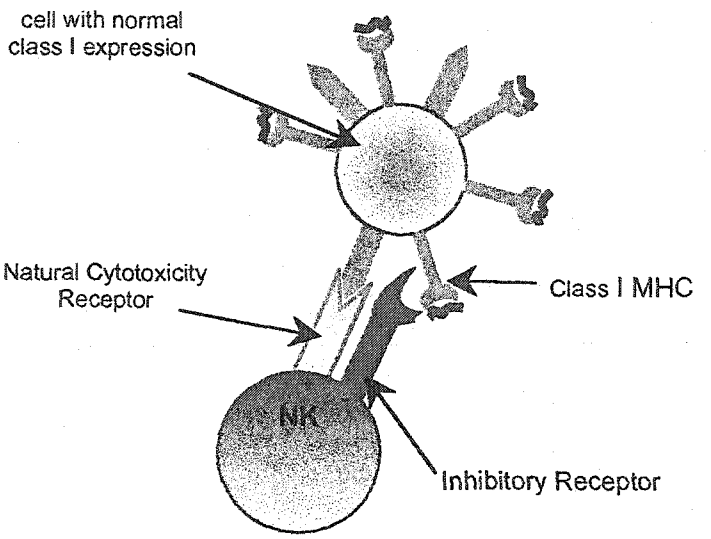

FIGURE 1-1. The missing self hypothesis. A natural cytotoxicity receptor generates signals leading to NK cell activation upon binding to its target cell ligand. An inhibitory receptor suppresses natural cytotoxicity when it engages its class I ligand on target cells. When target cell surface expression of the inhibitory receptor s class I ligand is deficient, NK cell activation signals predominate and the target cell is lysed.

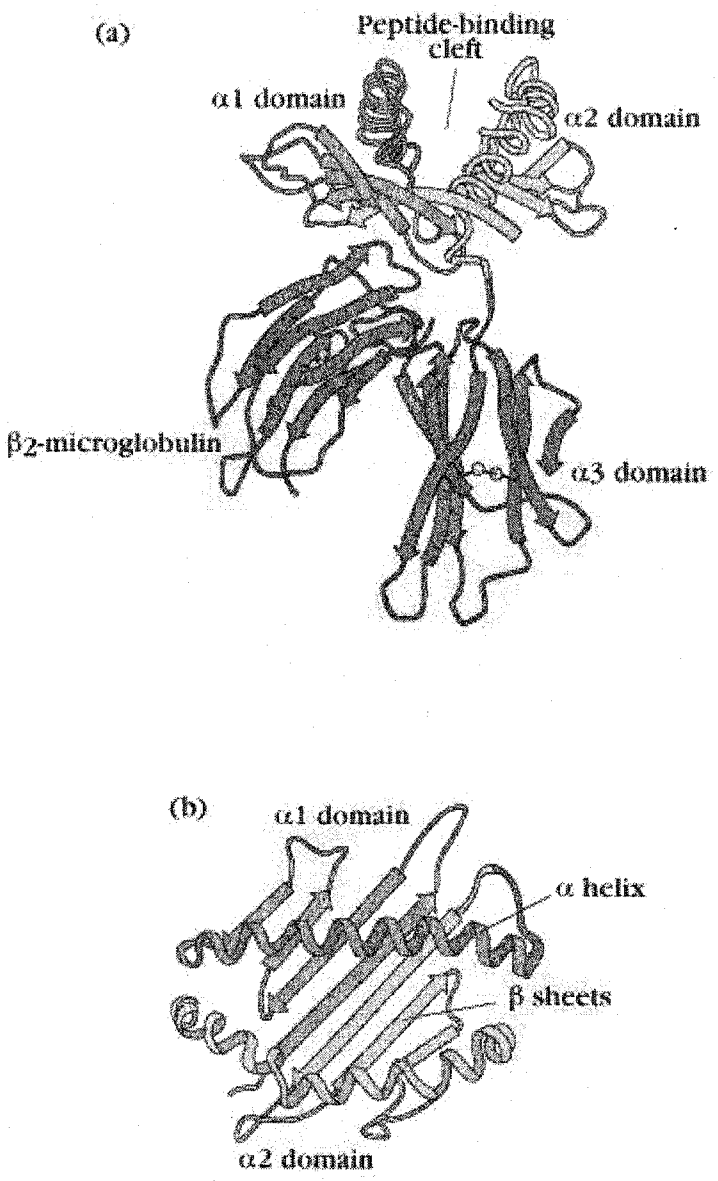

FIGURE 1-2. External domains of a class I MHC molecule based on x-ray crystallographic analysis. (a) Side view in which the β strands are depicted as thick arrows and the α helices as spiral ribbons. Disulfide bonds are shown as two interconnected spheres. (b) The α1 and α2 domains as viewed from top, showing the peptide-binding cleft consisting of a base of antiparallel β strands and sides of α helices. From Goldsby, Kindt, and Osborne (eds.), *Kuby IMMUNOLOGY, 4th Edition*, page 179 (2000).

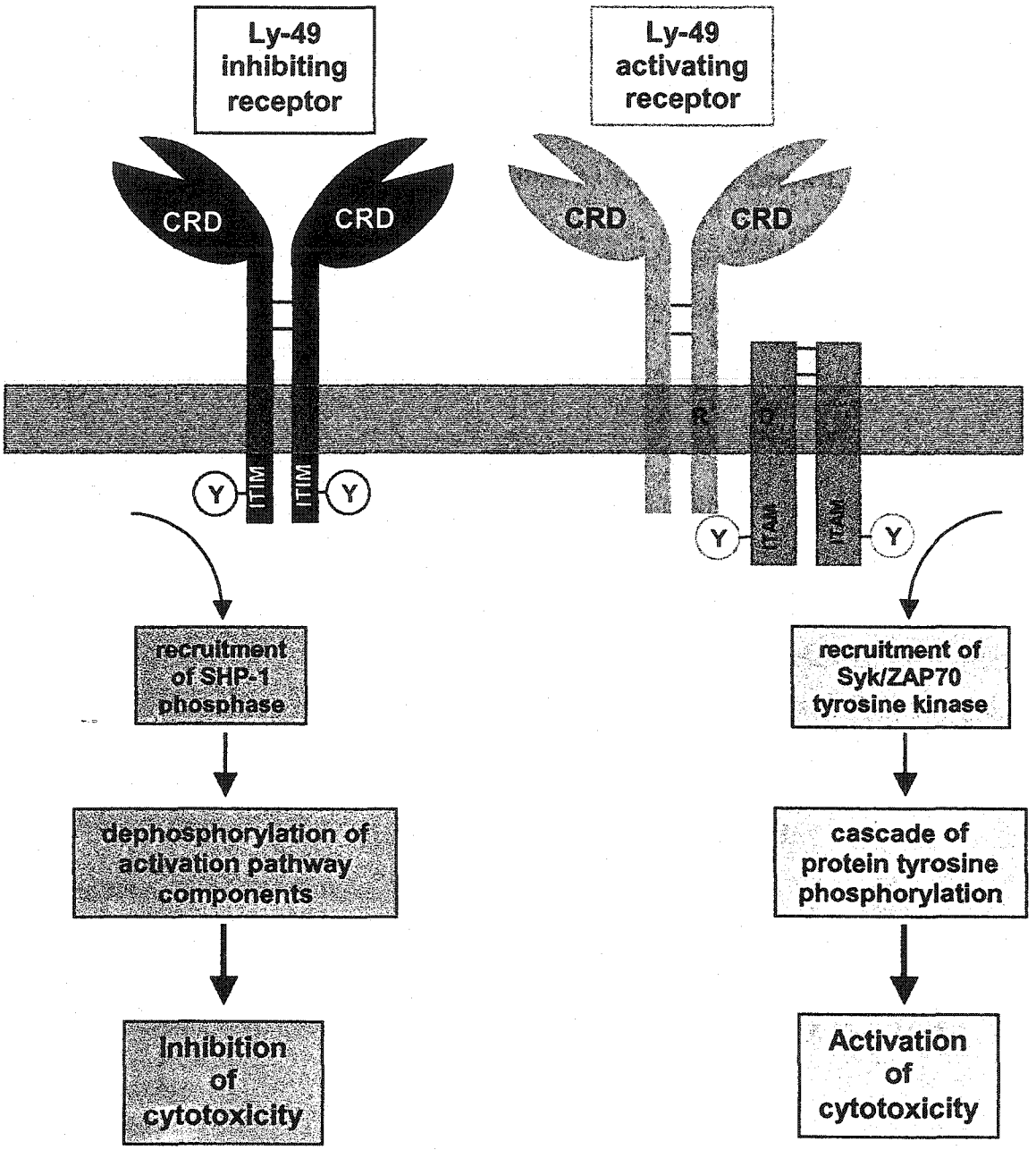

FIGURE 1-3. Structure and function of inhibitory and activating Ly-49 receptors. Binding of class I to an inhibitory Ly-46 receptor results in tyrosine phosphorylation of its ITIM, which recruits the SHP-1 phosphatase and results in cytotoxicity inhibition. Activating Ly-49 receptors lack ITIMs, and associate with the DAP12 adaptor protein through a charge-charge interaction in their transmembrane domains. Class I recognition by an activating Ly-49 receptor causes tyrosine phosphorylation of the ITAMs in the associated DAP12, which allows recruitment of Syk or ZAP70 and subsequent NK cell activation.

CHAPTER II

LY-49P ACTIVATES NK-MEDIATED LYSIS BY RECOGNIZING H-2D^{d} [1,2]

Elizabeth T. Silver, Dong-Er Gong, Chew Shun Chang, Abdelaziz Amrani, Pere Santamaria and Kevin P. Kane

A. Introduction

Ly-49 molecules are disulfide-bonded homodimeric type II transmembrane proteins belonging to the C-type lectin receptor superfamily (1). Ly-49 receptors are expressed on murine NK cells and certain subsets of murine T cells (2-4). Multiple Ly-49 receptors can be expressed simultaneously by an individual NK cell (5,6). A number of Ly-49 family members recognize class I MHC molecules, including Ly-49A, C and G and serve to inhibit NK function (7-9). Inhibitory Ly-49 receptors, like CD94/NKG2A and a variety of Ig-domain containing receptors, inhibit NK function by recruiting the

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SH2 domain-containing tyrosine phosphatase SHP-1 to disrupt tyrosine kinase dependent membrane proximal signaling events (10). Following inhibitory Ly-49 receptor engagement, phosphorylation of tyrosine within an immunoreceptor tyrosine-based inhibitory motif (ITIM) present on the cytoplasmic tail of each receptor subunit facilitates binding and activation of the SHP-1 phosphatase (10). Ly-49 receptor expression on NK cells can be modulated by class I MHC expression. For example, expression of the H-$2D^d$ ligand for Ly-49A *in vivo* can reduce the expression of Ly-49A on NK cells and correspondingly influences the class I density threshold for regulating NK lytic activity against H-$2D^d$-expressing targets (11). The purpose of inhibitory Ly-49 receptors appears to be to inhibit potentially autoaggressive NK function when "self" class I MHC expression on adjacent cells is in a normal density range. However, should the class I density drop significantly as a result of a disruption in class I expression, due to viral infection for example, Ly-49 inhibition is reduced or discontinued for lack of sufficient ligand, then NK lysis can ensue. This is the "missing self" hypothesis first proposed by Kärre and co-workers and functions as a paradigm for NK inhibitory receptor function (12).

The Ly-49 family also includes Ly-49D and H. These receptors lack ITIMs, do not recruit SHP-1 and are unable to function as inhibitory receptors. Instead, they associate with the disulfide-bonded immunoreceptor tyrosine based activation motif (ITAM)-containing signaling adapter molecule DAP12 and can stimulate transmembrane signaling initiated by tyrosine phosphorylation events (13,14). Association of Ly-49D or H with DAP12 is through a charge interaction between a conserved arginine in the transmembrane segment of Ly-49D and H, and a glutamic acid in the transmembrane segment of DAP12 (13). Engagement of Ly-49D or Ly-49H on NK cells with FcR-

mediated target cell bound antibodies, induces NK-mediated target cell lysis, by the mechanism of reverse antibody dependent cell-mediated cytotoxicity (rADCC), (15,16). Ly-49D has been shown to recognize the H-2D^d class I MHC molecule and this event results in NK activation (17-20). Thus, an activating Ly-49 receptor recognizes a class I MHC molecule. The Ly-49D$^+$ NK subset of some strains of mice have also been shown to mediate MHC-specific and Ly-49D-dependent allogeneic bone marrow rejection (21). These findings potentially complicate interpretation of the "missing self" hypothesis since they would not be readily predicted within the context of this model. Recently, the Ly-49D molecule was also shown to recognize undefined xenoantigens, resulting in NK lysis of certain xenogeneic target cells (22,23). Although able to stimulate NK cells, the role of activating Ly-49 receptors in regulating NK cell function remains unclear.

The Ly-49 family may be substantially larger than the commonly known members, Ly-49A through I. Complex Southern blot hybridizations obtained with Ly-49 specific probes using genomic DNA from inbred mouse strains suggest that additional Ly-49 family members may remain to be identified and characterized (24). Recently, genes or gene segments of five potential additional Ly-49s, tentatively designated J, K, L, M and N have been identified in the C57BL/6 (B6) genome (25). In addition, two Ly-49 cDNAs, one encoding an inhibitory receptor designated Ly-49O and another encoding an apparent activating receptor designated Ly-49P were recently cloned from the 129 mouse strain (26). The Ly-49P receptor is closely related to Ly-49A in its carbohydrate recognition domain (CRD), associates with DAP12 and triggers transmembrane signaling (26). These results suggested that Ly-49P may serve as an activating receptor. In this report, we describe the independent cloning of Ly-49P from non-obese diabetic (NOD) and non-obese diabetes resistant (NOR) NK cells and provide evidence that it can activate

NK cell cytolytic activity by recognizing H-2D^d. Prior to this report, Ly-49D was the only activating member of the Ly-49 family shown to recognize a class I MHC molecule. Thus, Ly-49P joins Ly-49D in this capability. Understanding the diversity, ligand specificity, activation potential and expression of activating Ly-49 members may provide insight toward understanding their function(s).

B. Materials and Methods

Animals

Five to eight week old female BALB/c (H-2^d), C57BL/6 (H-2^b), NOD (H-2K^d,D^b), NOR (H-2K^d,D^b), B10.BR (H-2^k), B10.D2 (H-2^d), B10.S (H-2^s), BALB.B10 (H-2^b) and BALB.K (H-2^k) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Additional five to eight week old NOR and NOD mice were obtained from the University of Alberta mouse breeding facility.

Hybridomas and antibodies

Hybridomas producing the following antibodies: A1(IgG$_{2a}$), anti-Ly-49A (27); MK-D6(IgG$_{2a}$), anti-IA^d (28); 11-4.1(IgG$_{2a}$), anti-H-2K^k (29); 14-4-4S(IgG$_{2a}$) anti-IE^k (30); BBM.1(IgG$_{2b}$), anti-human beta-2 microglobulin (31); B27M1(IgG$_{2a}$), anti-HLA-B7,27 (32); Y3(IgG$_{2b}$) anti-K^b (33); 34-2-12S(IgG$_{2a}$) anti-H-2D^d (α3 domain) (34,35); 34-5-8S(IgG$_{2a}$), anti-H-2D^d (α1/α2 domain) (34,35) and 2.4G2(rat IgG$_{2b}$) anti-mouse Fc-γ receptor (36); were obtained from ATCC (Manassas, VA) except A1, which was obtained from Dr. James Allison (University of California at Berkeley, Berkeley, CA). Antibodies were prepared by ammonium sulfate precipitation and PBS dialysis of tissue

culture supernatants obtained from hybridomas grown in protein free hybridoma medium. Purified G28 (IgG_{2a}) anti-rat CD8 (37), F23.1(IgG_{2a}) anti-mouse Vβ 8.1 and 8.2 T cell receptors (38,39), DX5 (rat IgM, phycoerythrin-coupled) which recognizes NK cells (3), 145-2C11 (hamster IgG, PerCP-coupled) which recognizes the CD3ε chain (40), and isotype control antibodies, G155-178,(mouse IgG_{2a}, fluorescein-coupled), anti-TNP, A19-3 (hamster IgG) anti-TNP, R4-22 (rat IgM, phycoerythrin-coupled) of unknown specificity, and A23-1, (rat IgG_{2c}) of unknown specificity were purchased from Pharmingen (San Diego, CA). The Ly-49A-reactive YE1/48 (rat IgG_{2c}) antibody (24), was kindly provided by Dr. Fumio Takei (Terry Fox Laboratories, Vancouver, B.C.) Tissue culture supernatants were also obtained from hybridomas grown in RPMI, 8% heat-inactivated FCS, 20mM Hepes, 2mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin for use in cell surface staining. FITC-coupled rat anti-mouse IgG, goat anti-rat IgG, and mouse anti-rat IgG were purchased from Jackson ImmunoResearch Labs (West Grove, PA).

Rat tumor cell lines

RNK-16, a spontaneous F344 rat strain NK cell leukemia cell line (41) was kindly provided by Dr. Mary Nakamura at the University of California at San Francisco. The RNK-16 cells were maintained in RPMI supplemented with 10% FCS, L-glutamine, penicillin, streptomycin and 5×10^{-5} M 2-mercaptoethanol (RNK medium). YB2/0 is a rat myeloma (42) we obtained from ATCC (Rockville, MD) and it was maintained in RPMI supplemented with L-glutamine, penicillin, streptomycin and 1mM sodium pyruvate (YB2/0 medium).

COS-7 cells

COS-7 SV40 transformed African green monkey kidney cells were provided by Dr. John Elliott (University of Alberta, Edmonton, AB, Canada).

PCR cloning of Ly-49 transcripts

Interleukin-2 (IL-2)-activated NK cells were prepared from spleen cells harvested from six-to ten week-old female mice as described by Smith and co-workers (2). Total cellular RNA was isolated from NOD and NOR IL-2-activated NK cells with TRIzol reagent (Life Technologies, Burlington, ON), and reverse-transcribed using SuperScript II (Life Technologies) with an oligo-dT primer. *Ly-49* transcripts were amplified with Advantage cDNA Polymerase (Clontech, Palo Alto, CA) using the sense cloning primer 5'CCCAAGATGAGTGAGCAGGAGG3' and the antisense cloning primer 5'GAGAGTCAATGAGGGAATTTATCC3'. The PCR products were purified using QIAquick spin columns (Qiagen Inc., Santa Clarita, CA) and directly ligated into the TA cloning vector p123T (Mo Bi Tec, Gottingen, Germany). Individual cDNA clones were divided into two groups: ITIM-encoding clones which could be PCR-amplified using the antisense cloning primer with the sense primer 5'GGAGACTCAAGGGCCCAGAG3', or ITIM-lacking clones which could be PCR-amplified by using the antisense cloning primer with the sense primer 5'CTRRAAAAGCT GGCCTCAGAGTY3' (where R=A/G and Y=C/T).

To verify the nucleotide sequences of the ends of the open reading frames and to obtain sequence of the non-coding gene flanking regions for cloning, we performed 5'- and 3'-rapid amplification of cDNA ends (5'- and 3'-RACE) for NOR *Ly-49A* and *Ly-49P*. Poly A^+ RNA was isolated from total RNA obtained from IL-2-activated NK cells using

Oligotex spin columns (Qiagen) and used to synthesize a cDNA library with the Marathon cDNA Amplification Kit (Clontech). The following gene-specific primers were used: for *Ly-49P* 5'-RACE, 5'CTTGAGGAGACGCTGAAGCCTAG3'; and for *Ly-49P* 3'-RACE, 5'GCCAGC TTTTCTAGGCTTCAGC3'. *Ly-49A* required nested primers: for 5'-RACE, 5'TGGTGAGATTTCATAAATCTGCAGGAT3' followed by 5'TGGCTACAGAAGGTGTTCATTCCAC3'; for 3'-RACE, 5'TCCTGCAGATTTATGAAATCTCACCAT3' followed by 5'ATGAACTTCCAGTGGAATGAACACCT3'. With the sequence data thus acquired, we designed the following cloning primers to obtain both NOD and NOR full length cDNA clones by RT-PCR: for *Ly-49A*, 5'TTCCTCCACCAGAACCACTTCTTG3' (sense) and 5'AGAAGATCTGTCCAGTCTCAACA GGG3' (antisense); for *Ly-49P*, 5'TTTAAAAGAGAACATACTCTACATCCTC3' (sense) and the same antisense primer as *Ly-49A*. *Ly-49D* was cloned by RT-PCR from NOD and NOR first strand cDNA using the same primers and conditions as were used to previously clone *Ly-49D* from C57BL/6 cDNA (43). Sequencing reactions were performed using a dideoxy terminator method and analyzed on an ABI 373A automated sequencer (Applied Biosystems, Foster City, CA).

Transfections

COS-7: COS-7 cells were grown in Opti-MEM I (Life Technologies) containing 5% heat-inactivated FCS (Medicorp, Montreal, QC, Canada). DNA containing the coding regions of *Ly-49A* and *Ly-49P* cDNA were inserted into the *Xba*I-*Eco*RI sites of the mammalian expression vector pCI-neo (Promega, Madison, WI). Sequence encoding the mature form of the mouse DAP12 protein was inserted into the pFLAG-CMV-1

expression vector (Sigma-Aldrich, Oakville, ON) to create an epitope-tagged chimeric protein. Vectors were then transfected into COS cells using LipofectAMINE (Life Technologies).

YB2/0 cells: A cDNA encoding H-2D^d cloned from S49.1 T lymphoma cells was inserted into the expression vector pCDNA3 and electroporated into YB2/0 using 180 mV and 960 μFD. A chimeric class I molecule, referred to as K^b/D^d, was produced by ligating the α1/α2 domain portion of H-2K^b to the α3, transmembrane and cytoplasmic portion of H-2D^d at the common FspI site. This chimeric cDNA was inserted into the expression vector pCI-neo and electroporated into YB2/0, as above. Transfected cells were immediately cloned in 96-well microtiter plates in YB2/0 growth medium supplemented with 1 mg/ml of G418 for drug selection (Life Technologies).

RNK cells: The coding region of *Ly-49P* was inserted into the *Xho*I-*Xba*I sites of the bicistronic expression vector BSRαEN which was generously provided by A. Shaw, Washington University, St. Louis, MO and transfected into RNK-16 cells using the protocol described by Nakamura et al. (10). Four million cells were transfected with 20 μg of linearized plasmid by electroporation at 180 mV and 960 μFD. Transfected cells were cloned in 96-well microtiter plates in RNK medium supplemented with 1 mg/ml G418 for drug selection.

Preparation of nylon wool nonadherent spleen cells and generation of IL-2-activated LAK.

Single cell suspensions of spleen cells from five to seven week old C57BL/6 and NOD mice were prepared with tissue grinders. After osmotic lysis of red blood cells, the cell suspensions were passaged over nylon wool columns. The nylon wool nonadherent

(NWNA) spleen cells were either analyzed immediately by FACS or cultured for six days at a concentration of $2x10^6$ cells/ml in RPMI-1640/10%FCS supplemented with sodium pyruvate, nonessential amino acids, 5×10^{-5} M 2-mercaptoethanol and 1000 U/ml human recombinant IL-2 expressed in and isolated from *E. coli*, prior to FACS analysis.

Flow cytometry

Approximately 48 hours after transfection, COS-7 cells were incubated for 30 minutes with A1 or isotype control mAb MK-D6. The cells were washed with phosphate buffered saline (PBS), incubated with FITC-labeled secondary antibody, washed again with PBS and then fixed with 2% formaldehyde for analysis on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). YB2/0 cells, as well as the H-$2D^d$ and H-$2K^b/D^d$ transfectants of YB2/0 were incubated with either rat IgG (Sigma) or normal rat serum before staining with the mAbs 34-2-12S or 14-4-4S, followed by FITC-conjugated rat anti-mouse IgG. YB2/0 transfectants expressing the chimeric class I molecule H-$2K^b/D^d$ were also analyzed by FACScan with the Y3 H-$2K^b$-specific mAb and the BBM.1 isotype control after blocking with normal rat serum, followed by FITC-conjugated secondary rat anti-mouse IgG and formaldehyde fixation. RNK-16 transfectants were incubated with normal rat serum for 30 minutes before addition of the mouse IgG_{2a} mAbs A1 and B27M1, followed by FITC-conjugated rat anti-mouse IgG, then fixed and analyzed by FACScan. Cells were gated for forward and side scatter.

Three-color analysis of NWNA cells: freshly isolated NWNA spleen cells, or 6 day IL-2 activated NWNA spleen cells were preincubated with normal mouse serum to block nonspecific antibody binding to Fc receptors before antibody incubation. Antibodies used for analysis were PerCP-coupled 2C11 (anti-mouse CD3ε),

phycoerythrin-coupled DX5 (anti-mouse pan-NK cell), and either fluorescein-coupled A1, or YE1/48 with a fluorescein-coupled mouse anti-rat second antibody. Isotype control antibodies used were A19-3-PerCP, R4-22-phycoerythrin, G155-178-fluorescein, and A23-1. Cells were gated for forward and side scatter.

Generation of Concanavalin A-activated blast target cells

Concanavalin A-activated T cell blasts were prepared from spleen cells of various mouse strains. Fifteen million spleen cells were cultured at $5.0x10^6$ cells/ml in RPMI with 10% heat-inactivated FCS, 2-mercaptoethanol and 3.0 μg/ml Con A for 48 hr. Blast cells were recovered either after three washes in RPMI medium or following Ficoll-Hypaque separation.

Cytotoxicity assays

Target cells were labeled at 37°C with 100 to 150 μCi of $Na^{51}CrO_4$ (^{51}Cr) (Mandel/NEN Life Science Products, Guelph, ON), for 1 hr if tumor cells, or 1.5 hr if Con A blast cells. Following extensive washes, $1x10^4$ ^{51}Cr-labeled target cells were incubated for 4-5 hr at 37°C in V-bottomed microtiter plates with RNK-16 cells or RNK-16 cells transfected with Ly-49P at various effector to target ratios in triplicate. After the incubation, plates were centrifuged for 5 min and 100 μl of supernatant was removed and counted in a gamma counter. The percent specific lysis was determined as [(experimental release - spontaneous release)/(maximum release - spontaneous release)] x 100. To perform the reverse antibody dependent cellular cytotoxicity (ADCC) experiments, untransfected RNK-16 cells and the 1B9 Ly-49P transfectant of RNK-16 were

preincubated for 15 min with 5 μg/ml of the A1, G28, F23.1 antibodies or medium alone prior to addition of FcR-expressing YB2/0 target cells and subsequent 4 hr cytotoxic assay as described. For antibody inhibition experiments, antibodies were incubated with soluble protein A (PA) [(2 μg per 10 μg of mAb), Sigma, St. Louis, MO)] for 30 min prior to addition to effector cells or target cells. Effector cells or target cells were preincubated with the mAb and protein A for 15 min prior to the cytotoxicity assay. The mAb were present throughout the cytotoxicity assay at a final concentration of 5 $\mu g/10^6$ effector cells or 5 μg/ml when the mAb was directed against a target cell antigen. All cytotoxicity assays were repeated a minimum of three times.

C. Results

Predicted amino acid sequences of Ly-49A, Ly-49D and Ly-49P of different mouse strains.

The Ly-49 family includes both activating and inhibitory receptors (1). Collectively, Ly-49 molecules may contribute to a balance of positive and negative signals that control murine NK activities, as well as those of certain subsets of T cells (3,7,8,9,18). Most studies of Ly-49 gene family members have used the B6 strain as a prototype. It is clear from recent studies, however, that the diversity and expression of Ly-49 members can differ among strains (44). Furthermore, complex hybridization patterns obtained in Southern blot analyses of genomic DNA from multiple inbred mouse strains using *Ly-49A* and *Ly-49C* cDNA probes suggest that additional Ly-49 members may exist (24). We chose to examine Ly-49 receptor expression and function in a mouse

strain other than B6, one in which immune dysregulation can occur, resulting in spontaneous disease. The NOD mouse spontaneously develops autoimmune disease at an early age (45), while the NOR mouse does not, although it shares many, but not all, NOD genetic loci (46). We designed oligonucleotide primers to clone cDNAs encoding putative activating and inhibitory Ly-49 family members by RT-PCR, and used RNA obtained from IL-2 activated NK cells of NOD and NOR mouse strain origin as sources of transcripts. In addition to cDNAs encoding well characterized Ly-49 family members, we also cloned a non-inhibitory Ly-49 family member that possessed high amino acid identity in its CRD with the inhibitory Ly-49A molecule. Sequence identities with a newly described activating Ly-49 present in the 129 mouse strain, designated Ly-49P by Makrigiannis et al. (26), indicate that we have cloned the NOD strain allele of Ly-49P. The NOR strain expresses Ly-49P identical in sequence to Ly-49P^{NOD}.

We also cloned a single cDNA sequence from both NOD and NOR NK cells encoding a novel allele of *Ly-49A*, since it has a very high identity of 99.1% and 99.6% on the nucleotide level, and 98.9% and 99.2% on the amino acid level, with B6 and BALB/c *Ly-49A* alleles, respectively (data not shown, Fig. 2-1A). The NOD (and NOR) *Ly-49A* allele is closer in sequence to both B6 and BALB/c alleles than they are to each other, 99.0% nucleotide (not shown), and 98.1% amino acid identities (Fig. 2-1A). The predicted Ly-49P^{NOD} amino acid sequence lacks an ITIM sequence and thus is not an inhibitory receptor, yet a comparison of the Ly-49P^{NOD} sequence with Ly-49A^{B6} indicates an amino acid identity of 83.3% for the entire molecule and 92.9% for the CRD (Fig. 2-1A). Similarly, a comparison of the Ly49P^{NOD} sequence with Ly-49A^{NOD} shows an overall amino acid identity of 82.5%, and 92.1% for the CRD region (Fig. 2-1A). In addition to the full length Ly-49P we have designated P1, there are two other major PCR

products amplified with Ly-49P cloning primers from NOD IL-2 activated NK cells. These smaller products are apparent splice variants of Ly-49P encoding in-frame transcripts containing the complete CRD region. Using the gene organization of Ly-49A as a reference (47), one Ly-49P variant lacks exon 4, while the other omits exons 3 and 4 (Fig. 2-1A). We designate the three Ly-49P splice variants by size as P1, P2 and P3. The first amino acid residue encoded by exon 5 of the P2 splice variant is predicted to differ from P1 and P3. This difference is a common result when two or more different RNA upstream donor sites are spliced to the same acceptor site. This occurs in transcripts of many genes including CD94 and NKG2B (48,49). In the case of P2, fusion of the first base of the codon ATT (Ile) at the end of exon 3 to the second and third bases of the codon GGC (Gly) at the beginning of exon 5 form the codon AGC (Ser) without interruption of the established reading frame. For the P3 splice variant, the first base of the codon GTG (Val) at the end of exon 2 is fused to the second and third bases of the codon GGC (Gly) at the beginning of exon 5 to reform the glycine codon at the start of the CRD, again without interruption of the established frame. We have been unable to clone a Ly-49P-related cDNA from IL-2-activated B6 NK cell RNA with primers used for cloning Ly-49P^{NOD}. It appears that either Ly-49P does not exist in the B6 genome, is not expressed, or is divergent in sequence from Ly-49P^{NOD}.

A comparison of the nucleotide and amino acid sequences of Ly-49P^{129} with Ly-49D of B6 origin suggested that Ly-49P transcripts are not derived from alleles of Ly-49D, but are encoded by a separate gene (26). We have confirmed this by sequencing Ly-49D cDNA cloned from NOD mice, indicating the existence of separate genes in the same mouse strain encoding Ly-49D and Ly-49P (Fig. 2-1B). Ly-49D cloned from NOR mice is identical in sequence to Ly-49D^{NOD}. High nucleotide (98.5%) and amino acid

sequence identity (96.6%) between Ly-49D^{NOD} and Ly-49D^{B6} confirm that these are very likely to be alleles (data not shown, Fig. 2-1B). The nucleotide and amino acid sequence differences between Ly-49D^{NOD} and Ly-49P^{NOD} are 84.8% and 85.6%, respectively (not shown and Fig. 2-1B), indicating that these are unlikely to be alleles. We are confident of the nucleotide and predicted amino acid sequences on the very ends of Ly-49P^{NOD} since we performed 5' and 3' RACE reactions to determine them, whereas sequences at the ends of Ly-49P^{129} reported previously (26), were primer derived and thus may contain minor sequence differences; nevertheless, a comparison of the entire Ly-49P^{NOD} and Ly-49P^{129} nucleotide and predicted amino acid sequences, 98.2% and 95.4% identity, respectively, strongly supports the conclusion that these are indeed alleles. There are only fourteen nucleotide differences found between these two cDNAs, resulting in twelve amino acid changes (data not shown and Fig. 2-1B). Another notable aspect of Ly-49P relates to potential post-translational processing: whereas Ly-49A and Ly-49D each have three predicted N-linked glycosylation sites, Ly-49P^{129} has two and Ly-49P^{NOD} has only one (Fig. 2-1A and B). The influence of N-linked glycosylation on Ly-49 expression and function remains to be determined. Finally, consistent with other noninhibitory Ly-49 family members, Ly-49P^{NOD} possesses a charged residue (Fig. 2-1B), arginine, in its putative transmembrane segment that is required for activating Ly-49 receptors to associate with the ITAM-containing signaling adaptor protein DAP12 (13). The sequences of Ly-49A$^{NOD/NOR}$, Ly-49D$^{NOD/NOR}$ and Ly-49P$^{NOD/NOR}$ were not artifacts of RT-PCR since they were confirmed in clones derived from different RT-PCR reactions with different sets of primers, and genomic sequence of Ly-49P has been shown to exist (26).

The A1 and YE1/48 antibodies recognize Ly-49P^{NOD}

The A1 antibody was previously reported to recognize a polymorphic epitope expressed only by the B6 allele of Ly-49A, since it was shown not to react with other known alleles of Ly-49A or other known Ly-49 family members (24). Since the noninhibitory Ly-49P^{NOD} possesses high sequence identity with Ly-49A, particularly in the CRD where the A1 antibody is predicted to bind Ly-49A (24), the reactivity of the A1 antibody and a second Ly-49A-specific antibody, YE1/48, with Ly-49P^{NOD} was examined. We expressed Ly-49A^{B6}, Ly-49A^{NOD} or Ly-49P^{NOD} in COS cells for antibody staining. Ly-49A^{B6} reacted with the A1 and YE1/48 antibodies as did Ly-49A^{NOD} and Ly-49P^{NOD} (Fig. 2-2A). There was some expression of Ly-49P detected with both antibodies when Ly-49P is expressed in the absence of DAP12, however, coexpression of DAP12 substantially augments Ly-49P expression as indicated by enhance staining with both antibodies (Fig. 2-2A). No staining of COS cells with the A1 antibody was observed in the absence of Ly-49A or Ly-49P expression, or with expression of DAP12 alone (data not shown). Coexpression of DAP12 was necessary for optimal expression of Ly-49P^{NOD} (Fig. 2-2A), similar to Ly-49P^{129} (26). Thus, the A1 antibody does not exclusively react with the B6 allele of Ly-49A, but also recognizes the NOD allele of Ly-49A and a noninhibitory/putative activating receptor, Ly-49P.

Ly-49P can function as an activating receptor for cytotoxicity by RNK-16 cells

Cross-linking of Ly-49P^{129} results in transmembrane signaling including tyrosine phosphorylation and Ca^{++} flux (26), suggesting that it can function as an activating receptor. We were interested in the ability of Ly-49P to activate NK cytotoxicity. To this end, we transfected Ly-49P^{NOD} into the heterologous rat NK leukemia RNK-16 which

displays NK activity (10). RNK-16 has been used successfully to demonstrate the NK activating function of transfected Ly-49D (17) and 2B4S, an activating form of an Ig-domain containing receptor expressed by murine NK cells (50). This experimental system avoids the complication of other murine activating and/or inhibitory receptor coexpression and cross-reaction of the A1 antibody with Ly-49 receptors other than Ly-49P. We show three Ly-49P transfectant clones that were positive for A1 antibody staining, 1B9, 1A3 and 1G4, while RNK-16 was negative (Fig. 2-2B), indicating that the transfectants express Ly-49P^{NOD}. These transfectant clones expressed different mean densities of Ly-49P (Fig. 2-2B). Transfectant 1A3 expressed quite a high density of Ly-49P, suggesting that possessing only one predicted N-linked carbohydrate attachment site is not a limitation for proper folding and cell surface expression of this molecule. RNK-16 cells and transfectants constitutively express the rat CD8 molecule as indicated by G28 antibody staining (Fig. 2-2B, right panels and data not shown).

To determine whether Ly-49P can function as an NK activating receptor, we examined the ability of Ly-49P transfectants to perform reverse antibody dependent cellular cytotoxicity (rADCC). The rat myeloma YB2/0 expresses Fc receptors and can be rendered susceptible to lysis when bound to an antibody directed against heterologous NK activating receptors expressed on RNK-16 cells by transfection (17). YB2/0 is normally sensitive to RNK-16 lysis (Fig. 2-3A); however, upon transfection of RNK-16 with murine noninhibitory NK receptors such as Ly-49D (17) and Ly-49P^{NOD} (Fig. 2-3B), cytotoxicity is reduced substantially. This may be due to the heterologous activating receptor competing successfully for a limiting signaling component, such as the rat DAP12, with endogenous rat activating receptor(s) responsible for YB2/0 recognition and lysis. An antibody directed against Ly-49P, but not rat CD8, stimulated rADCC of the

FcR bearing YB2/0 cells by the Ly-49P RNK-16 transfectant 1B9 (Fig. 2-3B). In contrast, the A1 antibody was unable to enhance the cytotoxicity of untransfected RNK-16 cells even at lower effector to target ratios where cytotoxicity is limiting (Fig. 2-3A). An additional isotype control antibody, F23.1 also did not affect lysis of YB2/0 by either effector cell (Fig. 2-3A and B). These results indicate that Ly-49P can transmit stimulating signals resulting in NK-mediated cytotoxicity and can be considered an NK activating receptor.

The MHC dependence of Ly-49P stimulated RNK-16 lysis of Con A-activated T cell blasts

A number of inhibitory Ly-49 family members have been shown to recognize class I molecules as ligands, including Ly-49A, C and G in their apparent role of inhibiting NK cells unless there is reduction of self MHC expression (7-9). Much less is understood regarding the ligand specificity and role of activating Ly-49 members. Ly-49D is the only activating Ly-49 subfamily member demonstrated to recognize a class I MHC molecule (17). Since Ly-49P, like Ly-49D, is an activating Ly-49 receptor for which there are serological reagents, we attempted to determine whether Ly-49P also recognizes class I MHC molecules as ligands. We first compared the ability of RNK-16 cells and three Ly-49P transfectants to lyse NK susceptible Con A T cell blast target cells from five different inbred mouse strains, BALB/c, B6, B10.BR, B10.D2 and B10.S (Fig. 2-4A). Lysis of all five strains was negligible for RNK cells, but each of the Ly-49P transfected RNK-16 cells lysed BALB/c and B10.D2 Con A-activated blasts and the transfectant with the highest Ly-49P expression, 1A3, also lysed B10.BR blasts, but to a much lower extent (Fig. 2-4A). No lysis of C57BL/6 or B10.S congenic Con A blasts was

observed. Taken together, these results suggest that Ly-49P may recognize an H-2^d product and possibly an H-2^k product.

To further evaluate the importance of MHC products in Ly-49P recognition, BALB/c, BALB.B10 and BALB.K Con A blasts differing only at the MHC were used as targets. Although there is some lysis observed with all of the BALB congenics by RNK-16 cells, lysis by the Ly-49P transfectants appears to be entirely dependent on the MHC expressed by the Con A blast targets. Using Ly-49P transfectants, no lysis is observed with BALB.B10 targets, but significant lysis is found if the target has the H-2^d MHC (Fig. 2-4B). A low level of lysis is also observed with the BALB.K target cells in the case of the 1A3 transfectant (Fig. 2-4B). We conclude from these experiments that an H-2^d product, possibly K^d or D^d, is a potential ligand for the Ly-49P activating receptor. It is also possible that an H-2^k product may be a much lower affinity ligand as well. Finally, we examined whether Ly-$49P^{NOD}$ is capable of activating RNK-16 lysis of NOD and NOR Con A blasts. Although B10.D2 Con A blasts were lysed, no lysis of NOD or NOR Con A blasts was observed using the Ly-$49P^{NOD}$ transfectants (Fig. 2-4C). This suggests that the NOD/NOR MHC molecules, H-$2K^d$ and D^b, which are identical to the K^d and D^b alleles of BALB/c and B6 mice, respectively, are apparently not detectable ligands for Ly-$49P^{NOD/NOR}$. Considering all the results together, they suggest that Ly-49P, like Ly-49D, may recognize the MHC molecule, H-$2D^d$.

The foregoing results suggested that Ly-49P triggers RNK-16 mediated lysis of Con A blasts by recognizing H-$2D^d$. To more directly assess this possibility we attempted to block lysis mediated by the Ly-49 RNK transfectants, 1B9 and 1A3, by including the A1 antibody which recognizes Ly-49P, or the 34-5-8S antibody which recognizes the $\alpha 1/\alpha 2$ domain of H-$2D^d$ expressed by the Con A blast targets, in the

cytotolytic assays. Soluble protein A was used to bind the Fc portion of the antibodies to prevent ADCC. The A1 antibody, but not one directed against rat CD8 expressed by the RNK-16 cells, was able to completely block lysis of B10.D2 Con A blast targets by 1B9 (Fig. 2-5A) and 1A3 (Fig. 2-5B), indicating that Ly-49P is the receptor responsible for activating lytic potential of RNK-16 cells. In a similar fashion, the 34-5-8S antibody, but not an isotype control antibody 11-4.1, completely blocked lysis of the B10.D2 Con A blasts by a representative Ly-49P transfectant, 1B9 (Fig. 2-5C). Similar effects were observed with 34-5-8S but not 11-4.1 using the 1A3 transfectant and B10.D2 Con A blasts (data not shown). No effect of soluble protein A alone was observed in the assays (Fig. 2-5A to C). These results suggest that Ly-49P recognizes H-2D^d, possibly at a location within its α 1/α 2 domains, as has been reported for Ly-49A and Ly-49D (10,17,51,52).

Recognition of the H-2D^d α1 α2 domain by Ly-49P stimulates cytotoxic function in RNK-16 cells

The previous antibody blocking experiments suggested that the α1/α2 domain of H-2D^d is recognized by Ly-49P. To directly assess this possibility we transfected rat YB2/0 cells with genes encoding either wild type H-2D^d, or a class I chimera referred to as K^b/D^d, in which the α3 domain of D^d remains but the α1 and α2 domains of K^b are substituted for the same domains of D^d, for use as targets of Ly-49P transfected RNK-16. Two D^d and two K^b/D^d transfectant clones are shown to express comparable densities of class I using the 34-2-12S antibody which recognizes an epitope on the D^d α3 domain shared by both molecules (Fig. 2-6, upper panels). Expression of the K^b α1/α2 domain on

the K^b/D^d chimeric YB2/0 transfectants is confirmed by Y3 antibody staining (Fig. 2-6, lower panels). A substantial increase in lysis is observed with the wild type D^d YB2/0 transfectants over that observed with YB2/0 using a representative Ly-49P transfectant of RNK-16, 1B9 (Fig. 2-7). No increase in lysis over that found with YB2/0 was detected using the YB2/0 transfected with the K^b/D^d chimeric gene (Fig. 2-7). These results indicate that Ly-49P can activate NK-mediated lysis as a consequence of interacting with the $\alpha1/\alpha2$ domain of H-$2D^d$. This specificity is shared with the inhibitory Ly-49A receptor and the activating receptor Ly-49D.

Expression of A1 and YE1/48 epitopes by NOD natural killer cells

We have demonstrated that NOD IL-2 activated NK cells express Ly-49A and Ly-49P at the RNA level and shown that Ly-49P can serve as an NK activating receptor by recognizing a class I MHC molecule. To determine whether these receptors may be expressed by NK cells of the NOD mouse, we performed three-color FACS analysis on freshly isolated NWNA and IL-2 activated NOD spleen cells. Because the NOD mouse strain does not express the NK1.1 epitope, we used the DX5 antibody to identify NK cells (3,44). $DX5^+CD3^-$ cells (Fig. 2-8, top panels) were analyzed for staining with YE1/48 and A1 antibodies (Fig. 2-8, middle and bottom panels). A substantial portion of freshly isolated NOD NK cells express A1 and YE1/48 recognized epitopes (Fig. 2-8, left panels), and two populations of YE1/48 and A1 positively stained fresh NOD NK cells apparently exist; a small population constituting 2.5-4% of total NK cells which are strongly stained and a larger population of more weakly stained cells, most effectively seen with the YE1/48 antibody, that constitute 50-80% of total NOD NK cells. This pattern contrasts with C57BL/6 NK cells where approximately 20% express A1 and

YE1/48 epitopes that uniformly stain with high fluorescence intensities, similar to those observed for the smaller $A1^+$ and $YE1/48^+$ NOD NK subset(s) (44, data not shown). The $DX5^+CD3^-$ IL2-activated NOD NK cells (Fig. 2-8, right panels) show similar numbers of cells staining with the A1 and YE1/48 antibodies compared to their freshly isolated NK counterparts, with perhaps one notable difference (Fig. 2-8). The IL-2 activated NOD NK expressing the YE1/48 and A1 recognized epitopes appear to fall into less discrete populations than fresh NOD NK cells, with increased numbers of cells demonstrating moderate to high levels of A1 and YE1/48 staining. Together, these results indicate that Ly-49A and/or Ly-49P are expressed at the surface of freshly isolated and IL-2 activated NOD NK cells. Since $Ly\text{-}49A^{NOD}$ and $Ly\text{-}49P^{NOD}$ are both recognized by YE1/48 and A1 antibodies, the staining pattern however, does not allow us to readily discern which cells express Ly-49A and/or Ly-49P.

D. Discussion

In this report we demonstrate that $Ly\text{-}49P^{NOD}$ is a receptor that activates NK lysis by recognizing a class I MHC molecule, $H\text{-}2D^d$. It is the second activating Ly-49 receptor that recognizes an MHC molecule, of two examined to date for ligand specificity. Although the "missing self" hypothesis is consistent with results obtained in studies of inhibitory Ly-49 receptors, it does not account for activating receptors which recognize class I MHC molecules. It would appear that any comprehensive model for Ly-49 regulation of NK cells must also account for activating Ly-49 receptors that function by recognizing MHC molecules.

McQueen et al. have described the existence of genes encoding putative activating Ly-49s designated K, L and N (25). Ly-49K and N have highest homology to Ly-49C in

the CRD, whereas the Ly-49L CRD most resembles Ly-49D (25). Ly-49K and N transcripts were found to contain premature stop codons and/or defective alternative splices and do not encode functional protein products (53). We show here that full length Ly-49P transcripts expressed in NOD and NOR mice can encode a viable protein product. This was also found to be the case for Ly-49P expressed in the 129 strain (26). We identified two additional alternatively spliced forms of Ly-49P in NOD/NOR NK cells. One splice variant, P2, encodes a Ly-49P protein that would lack the membrane proximal stalk portion, but is otherwise not different from full length Ly-49P. Transcripts encoding other NK C-type lectin receptor subunits, such as NKG2A and NKG2C, can undergo identical splicing (48, 54, 55). The smallest splice variant, P3, removes a portion of the cytoplasmic domain, the transmembrane segment and the stalk region. This transcript encodes a protein that would probably remain cytosolic. Equivalent splice variants to P3 have been identified for several NK C-type lectin superfamily members (53). The P1, P2 and P3 messages appear to be expressed in approximately equivalent amounts judging from the intensity of PCR amplified products (data not shown). It remains to be determined whether proteins encoded by Ly-49P2 and Ly-49P3 transcripts are expressed as stable polypeptides and what function they may perform.

Strain 129 NK cells, although expressing transcripts for Ly-49P, do not express transcripts for Ly-49A (26). In contrast, C57BL/6 strain NK cells express Ly-49A but no transcripts for Ly-49P are found, (26) and data not shown. It is not clear whether the Ly-49A gene is absent in the 129 strain or simply silent, and the same question is pertinent for Ly-49P in the B6 strain. It could be speculated that the genes for Ly-49A and Ly-49P are found in separate mouse strains or expression of these two genes may be mutually exclusive in a strain specific manner. However, we were able to clone Ly-49A and Ly-

49P cDNAs from both NOD and NOR strain IL-2 activated NK cells, suggesting that Ly-49A and Ly-49P genes can reside in the same genome and can be expressed at the same time. We identified two subsets of freshly isolated NOD NK cells and possibly IL-2-activated NOD NK cells that stained with the YE1/48 and A1 antibodies, a small subset that stains relatively strongly and a larger subset that stains weakly. Since Ly-49A positive cells from several mouse strains stain strongly with these antibodies (44), it is possible that the strongly stained NOD NK subset we have observed, expresses Ly-49A. The level of YE1/48 staining observed with the weakly stained NOD NK cells reported here, resembles the weak level of YE1/48 staining previously reported with 129 strain NK cells which express Ly-49P, but not Ly-49A (26). By analogy, it is possible that the weakly staining NOD NK cells express Ly-49P. It is conceivable, therefore, that Ly-49A and Ly-49P may be expressed on separate fresh NOD NK subsets, but further studies will be necessary to resolve this possibility.

It has been suggested that the Ly-49P gene arose during evolution from recombination of two Ly-49 genes (26). This conclusion was based on a comparison of nucleotide sequences of Ly-49P, A and D (26). An interval within exon 4 of Ly-49P^{129} may have been a site of recombination between the inhibitory Ly-49A gene and an activating Ly-49 gene. In any event, Ly-49P DNA sequences exist in the mouse genome (26). Nucleotide sequences of Ly-49P^{NOD} transcripts are also consistent with some form of recombination (data not shown). Determining the identity of the activating Ly-49 donor gene contributing the 5' portion of the Ly-49P gene, however, may require full characterization of the activating Ly-49 gene repertoire. Activating Ly-49 genes unable to produce viable transcripts may be an additional source of such sequences for recombination (53). Recombination between Ly-49 genes may provide a mechanism for

creating additional functional diversity within the Ly-49 gene family. Since Ly-49A and Ly-49P genes can be expressed by the same mouse strain, it suggests that if recombination was the origin of Ly-49P, Ly-49A gene duplication may have occurred prior to the recombination event. Gene conversion is another possible mechanism by which Ly-49P arose without involving Ly-49A gene duplication.

Ly-49A and Ly-49D are receptors that recognize H-2D^d (10,17). Ly-49P shares amino acid sequence identities with both of these receptors, particularly with the CRD of Ly-49A, where there are only nine amino acid differences in this domain which consists of 127 residues (Fig. 2-1). Despite being highly related in sequence to Ly-49A and D, the specificity of Ly-49P was not assured. We have demonstrated that Ly-49P does indeed recognize H-2D^d in NK functional studies. It is possible that Ly-49P may also recognize an H-2^k product; however should this be the case, our data suggest that it is probably with substantially lower affinity. It is worth noting that Ly-49A has been reported to recognize D^k although less effectively than D^d (56,57), whereas Ly-49D can recognize D^d, but does not appear to be able to interact with H-2^k products (17,18). It is possible that Ly-49P may share the class I specificity of Ly-49A, but its ability to also recognize H-2^k products still remains to be confirmed. In our studies, Ly-49P^{NOD} did not recognize NOR or NOR Con A blasts, suggesting that this NK activating receptor, or this allele of Ly-49P, does not recognize self MHC molecules in the NOD mouse.

The crystal structure of Ly-49A bound to H-2D^d has recently been resolved to 2.3Å (58). The Ly-49A CRD folds into seven β strand and two α helix secondary structure elements. Given the high degree of sequence identity between Ly-49A and Ly-49P in the CRD, it is likely that the Ly-49P CRD would form a very similar structure. In the crystal complex, Ly-49A binds H-2D^d at two sites: site 1 straddles the α1/α2 domain

boundary; site 2 involves the cleft formed by the α3 domain, beta-2 microglobulin and the bottom of the platform supporting the peptide binding groove where a portion of CD8 has also been shown to bind (58). It remains to be determined whether site 2 on D^d participates in Ly-49A binding when in *trans*, i.e. during an interaction with D^d expressed on a target cell. We show that the D^d α1/α2 domain is required for Ly-49P recognition which is consistent with participation of site 1. In fact, Ly-49P conserves all twelve of the $Ly\text{-}49A^{B6}$ contact residues for this site, (58) and Fig. 2-1A. Ly-49P also conserves twenty-four of the twenty-five residues of Ly-49A that may interact with site 2 [(58) and Fig. 2-1A].

The NOD mouse strain, from which we cloned $Ly\text{-}49P^{NOD}$, has immunodeficiencies and develops type I diabetes (45, 59). Adoptive transfer experiments have demonstrated that T cells are required for the induction of diabetes in the NOD mouse (reviewed in 45). Natural killer cells, as well as T cells and B cells, are found in the cellular infiltrates of pancreatic insulitis in the NOD mouse, but the role of NK cells, if any, in the disease process remains unclear (60). Natural killer cells of NOD mice have been described as functionally deficient. This characteristic is attributed as a result of observations that NOD NK cells lyse the prototype NK target cell, Yac-1, less efficiently than NK cells of other mouse strains (61, 62). Determining the extent of the NOD NK deficiency will require a comprehensive analysis of NK lytic potential and cytokine production in response to additional target cell types. We have shown that NOD mouse NK cells express viable transcripts for the NK activator molecule Ly-49P, and subsets of NOD NK cells stain with antibodies reactive with Ly-49P and Ly-49A, suggesting that Ly-49P may be expressed at the cell surface of some NOD NK cells. Ly-49P and other NK activator molecules may be relevant for NOD NK recognition of targets other than

Yac-1. The identification of NK activator molecules and the development of serological reagents specific for these receptors may aid in the characterization of NOD NK functional efficiency, as well as determining the contribution, if any, of NK cells to the development of diabetes in the NOD mouse.

The NOD/SCID mouse has shown particular utility in studying human tumor growth and development as well as human hematopoiesis, since it accepts a broader spectrum of human xenografts than SCID or nude mice (59, 63). Human graft acceptance by NOD/SCID mice has been suggested to be due to the NK functional deficiency of the NOD mouse in combination with a lack of B cell and T cells related to the SCID mutation (59, 63). Mice lacking the common γ chain, which is required for signaling through a number of cytokine receptors, including the IL-2 receptor and IL-15 receptor, do not develop functional natural killer cells (64). Mice lacking the common gamma chain coupled with the SCID mutation accept various human xenografts much more readily than NOD/SCID mice (64). This difference may suggest that NOD NK cells have an ability, although possibly diminished relative to other strains, to reject xenografts. Since the NK activating receptor Ly-49D of C57BL/6 origin has demonstrated xenoreactivity against cells of rat and hamster origin, albeit not against the limited number of human target cell types tested as yet (22,23), perhaps Ly-49D, Ly-49P or additional NK activating Ly-49 receptors expressed by the NOD mouse, are responsible for some of the residual barriers to human xenografts exhibited by the NOD/SCID mouse.

Natural killer cells are not the only cells that express Ly-49 receptors, as some T cell subsets can also express these receptors (3). Natural killer T (NKT) cells, for example, express TCR using a conserved TCR alpha chain and are regulatory T cells that rapidly secrete large quantities of IL-4 or IFN-γ in response to glycolipid antigens

presented by the non-classical class I-like CD1 molecules (65). As the name implies, NKT cells express various receptors normally found on NK cells including NKRP.1 and Ly-49 molecules. Interestingly, NKT cell numbers are reduced in the diabetes-prone NOD mouse relative to other mouse strains and manipulations that substantially increase the number of NKT cells result in protection from diabetes (66, 67). Evidence provided by Exley et al. (68), suggests that the NK receptor NKR-P1A, expressed on human NKT cells, may function as a costimulator in NKT cell activation. It remains to be determined whether mouse NKT cells express activating Ly-49 family members and what role these receptors may play in NKT cell function.

The significance of Ly-49P expression in NOD and NOR mice with respect to immune regulation is unclear. However, it is perhaps notable that some of the pancreas-infiltrating T cells of both strains of mice express Ly-49P transcripts and the A1 epitope by FACS analysis (data not shown). Whether cells of NOD or NOR mouse tissues other than splenic T cells express ligands for Ly-49P, in normal or disease states, remains to be determined.

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F. Author Contribution to Data

Elizabeth Silver generated the C57BL/6, BALB/c, NOD and NOR cDNA libraries, cloned by RT-PCR the *Ly-49* transcripts reported in this chapter, performed the COS transient transfections, generated the stable Ly-49 and class I transfectant clones, and did the flow cytometric analyses for this study. Dong-Er Gong performed the cytotoxicity assays published in this study. Chew Shun Chang cloned the H-2D^d cDNA used in this study. Abdelaziz Amrani and Pere Santamaria provided the NOD and NOR CTL clones that initiated this study and from which a cDNA fragment of Ly-49P was initially obtained.

G. Erratum

The 129/J Ly-49P allele amino acid sequence in Fig. 2-1B is incorrect due to errors in the original GenBank sequence, accession number AF146570, which has been corrected. All corrections are within exons 3 and 4, and the correct alignment in the affected region is shown below. Overall nucleotide and amino acid identities between Ly-49P NOD and 129/J using the corrected sequence are 99.2% and 98.1%, respectively. These levels of identity are even higher than those stated in the original report and even more strongly support the conclusion in the paper that these are indeed alleles of the same gene.

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                    60         70         80         90        100        110
Ly-49P (NOD)  GILISLRLVI VAVLVTNIFQ YGQQKHELQE FLNHHNNCSI MQSDIKLKDE LLKKKSIECN
Ly-49P (129/J) ---------- ---------- ---------- ---------- --------N- ----------

                   120        130        140
Ly-49P (NOD)  LLESLNRDQN RLYSKTKTVL DFLQHTGRGD
Ly-49P (129/J) ---------- ---N------ ----------
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A

Exon 2 (Cytoplasmic Region)

```
Ly-49A (B6)      MSEQEVTYSMVRFHKSAGLQKQVRPEETKGPREAGYR
Ly-49A (BALB/c)  ----------------------------------------
Ly-49A (NOD)     ----------------------------------------
Ly-49P1 (NOD)    -------F-T---Q-TS---NR--L---LK--K--L-
Ly-49P2 (NOD)    -------F-T---Q-TS---NR--L---LK--K--L-
Ly-49P3 (NOD)    -------F-T---Q-TS---NR--L---LK--K--L-
```

Exon 3 (Cytoplasmic and Transmembrane Regions)

```
Ly-49A (B6)      RCSFHWKFIVIALGIFCFLLLVAVSVLAIK
Ly-49A (BALB/c)  ----------------------T-------
Ly-49A (NOD)     ----------------------T-------
Ly-49P1 (NOD)    V--VP-Q--------LIS-R--I-A--VTN
Ly-49P2 (NOD)    V--VP-Q--------LIS-R--I-A--VTN
Ly-49P3 (NOD)    ******************************
```

Exon 4 (Extracellular: Stalk Region)

```
Ly-49A (B6)      IFQYDQQKK*LQEFLNHHNNCSNMQSDINLKDEMLKNKSIECDLLESLNRDQNRLYNKTKTVLDSLQHT
Ly-49A (BALB/c)  ---------*---------------------------------------G----------
Ly-49A (NOD)     ---------*---------------------------------------G----------
Ly-49P1 (NOD)    ----G---HE---------I-----K----L--K-----N----------S-------F----
Ly-49P2 (NOD)    ************************************************************
Ly-49P3 (NOD)    ************************************************************
```

Exon 5 (Extracellular: CRD)

```
Ly-49A (B6)      GRGDKVYWFCYGMKCYYFVMDRKTWSGCKQTCQSSSLSLLKIDDEDEL
Ly-49A (BALB/c)  -------------------------------------------------
Ly-49A (NOD)     -------------------------------------------------
Ly-49P1 (NOD)    ------------R----------P-----------G------------
Ly-49P2 (NOD)    S-----------R----------P-----------G------------
Ly-49P3 (NOD)    ------------R----------P-----------G------------
```

Exon 6 (Extracellular: CRD)

```
Ly-49A (B6)      KFLQLVVPSDSCWVGLSYDNKKKDWAWIDNRPSKL
Ly-49A (BALB/c)  ---H-----------------------------
Ly-49A (NOD)     ---H-----------------------------
Ly-49P1 (NOD)    ----------V--I-----------S-V--G----
Ly-49P2 (NOD)    ----------V--I-----------S-V--G----
Ly-49P3 (NOD)    ----------V--I-----------S-V--G----
```

Exon 7 (Extracellular: CRD)

```
Ly-49A (B6)      ALNTRKYNIRDGGCMLLSKTRLDNGNCDQVFICICGKRLDKFPH
Ly-49A (BALB/c)  ------------------I--------------A--------
Ly-49A (NOD)     -------------------------------------------
Ly-49P1 (NOD)    ----------------------------------A--------
Ly-49P2 (NOD)    ----------------------------------A--------
Ly-49P3 (NOD)    ----------------------------------A--------
```

Figure 2-1A. Alignment of predicted Ly-49A, D, and P amino acid sequences from different mouse strains. *A*, Amino acid sequences of Ly-49A alleles from C57BL/6, BALB/c and NOD strains are aligned with NOD Ly-49P sequences. Exon assignments are based on the genomic organization of C57BL/6 Ly-49A. The ITIM sequence is boxed with dotted lines, and the putative transmembrane region is shown in bold. Dashes indicate sequence identity, asterisks indicate sequence gaps, and potential N-linked glycosylation sites are boxed with solid lines. The nucleotide sequences are available under the GenBank accession numbers AF218077 (Ly-49A^{NOD}), AF074456 (Ly-49A^{NOR}), AF218080 (Ly-49P1^{NOD}), AF218081 (Ly-49P2^{NOD}), AF218082 (Ly-49P3^{NOD}), AF074458 (Ly-49P1^{NOR}), AF074461 (Ly-49P2^{NOR}), and AF074462 (Ly-49P3^{NOR}).

B

```
                    10         20         30         40         50         60
Ly-49P (NOD)  MSEQEVTFST VRFQKTSGLQ NRVRLEETLK PRKAGLRVCS VPWQFIVIAL GILISLRLVI
Ly-49P (129/J) ---------- ---------- ---------- ---------- -S-------- ---------T
Ly-49D (NOD)  -T--KD---A ---H-S----- -EM--K--W- -Q-------- ---------- ----------
Ly-49D (B6)   -T---D---A ---H-S----- -EM--K--R- -E--R----- ----L----- ---------T

                    70         80         90        100        110        120
Ly-49P (NOD)  VAVLVTNIFQ YGQQKHELQE FLNHHNNCSI MQSDIKLKDE LLKKKSIECN LLESLNRDQN
Ly-49P (129/J) ----M----- --------K- --K------- -----N---- --N------D ----------
Ly-49D (NOD)  ----M----- ---------- --K------- -----N---- --N------- ----------
Ly-49D (B6)   ----M----- --------K- --K------- -----N---- --N------- ----------

                   130        140        150        160        170        180
Ly-49P (NOD)  RLYSKTKTVL DFLQHTGRGD KVYWFCYGRK CYYFVMDRKP WSGCKQTCQS SGLSLLKIDD
Ly-49P (129/J) ---N------ -S-------- --------M- ---------- ---------- ----------
Ly-49D (NOD)  I-CD--R--- -S-------V --------M- ---------- --R---S--N -S-T------
Ly-49D (B6)   I-CD--R--- -Y-------V --------M- ---------- --R---S--N -S-T------

                   190        200        210        220        230        240
Ly-49P (NOD)  EDELKFLQLV VPSDVCWIGL SYDNKKKDWS WVDNGPSKLA LNTRKYNIRD GGCMLLSKTR
Ly-49P (129/J) ---------- ---------- ---------- ---------- ---------- ----------
Ly-49D (NOD)  --------F- ----S----- ---------A -I--R----- ---T------ ----F-----
Ly-49D (B6)   ---------- ----S----- ---------A -I--R----- ---T------ ----F-----

                   250        260
Ly49P (NOD)   LDNGNCDQVF ICICAKRLDK FPH
Ly49P (129/J) ---------- ---------N ---
Ly49D (NOD)   ---NY---S- ----G----- ---
Ly49D (B6)    ---NY---S- ----G----- ---
```

Figure 2-1B. Alignment of predicted Ly-49A, D, and P amino acid sequences from different mouse strains. *B*, NOD and 129/J Ly-49P sequences are aligned with Ly-49D alleles of NOD and C57BL/6 strains. The arginine in the transmembrane region is shown in bold. Dashes indicate sequence identity, asterisks indicate sequence gaps, and potential N-linked glycosylation sites are boxed with solid lines. The nucleotide sequences are available under the GenBank accession numbers AF218080 (Ly-49P1^{NOD}), AF074458 (Ly-49P1^{NOR}), AF218078 (Ly-49D^{NOD}), and AF218079 (Ly-49D^{NOR}).

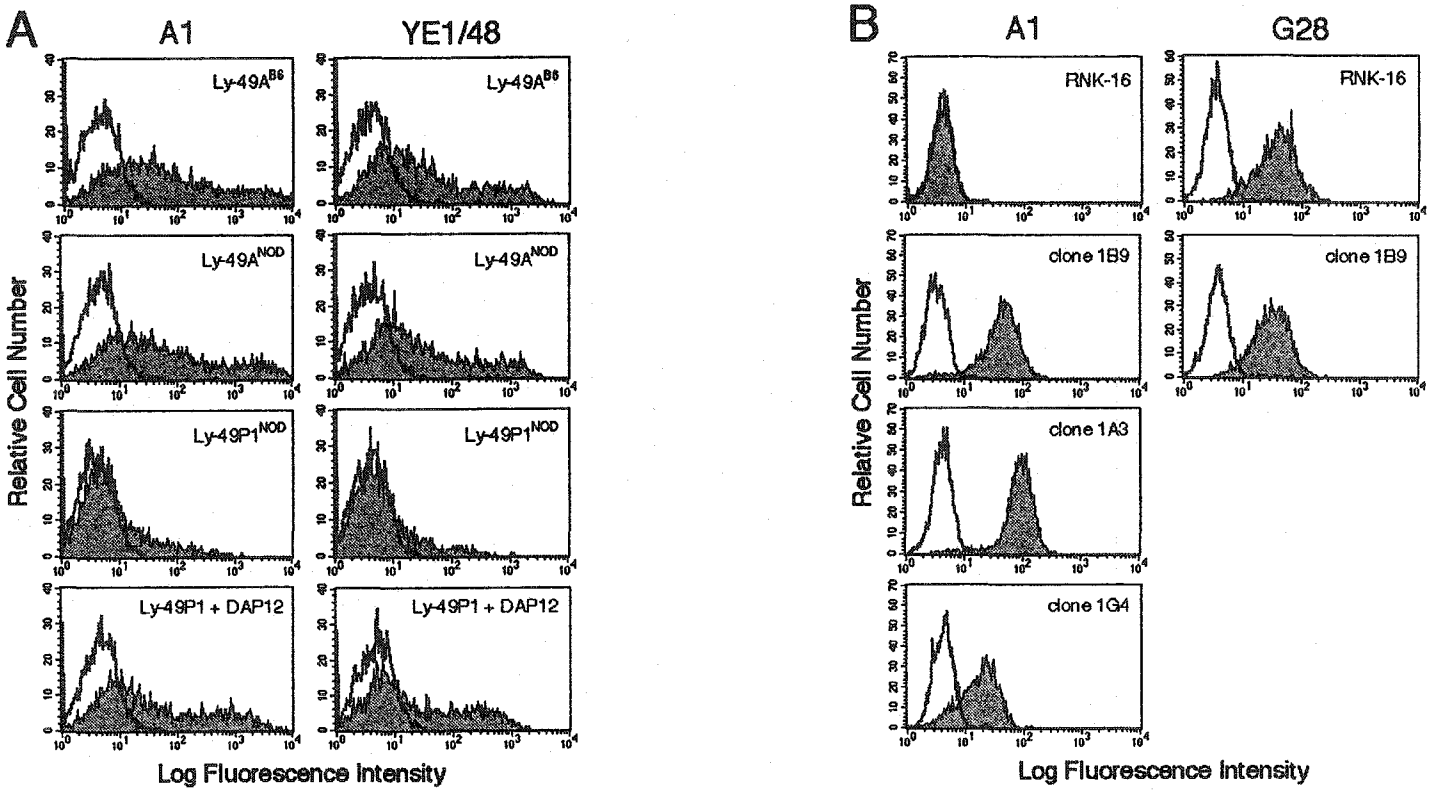

Figure 2-2. Ly-49P^{NOD} is recognized by the A1 and YE1/48 antibodies. *A,* FACS analysis of COS cells transfected with Ly49A^{B6}, Ly-49A^{NOD}, or Ly-49P^{NOD} alone or with murine DAP12, using the A1 or YE1/48 antibodies (shaded), or the isotype controls antibodies MK-D6 or 2.4G2 (unshaded). Binding was detected with fluorescein-coupled rat anti-mouse or goat anti-rat antibody, respectively. *B,* FACS analysis of RNK-16 (upper panels), and Ly-49P transfected RNK-16 clones 1B9 (second panels), 1A3 (third panel), and 1G4 (bottom panel) using the antibodies A1 or G28 (shaded), or the isotype control B27M1 (unshaded).

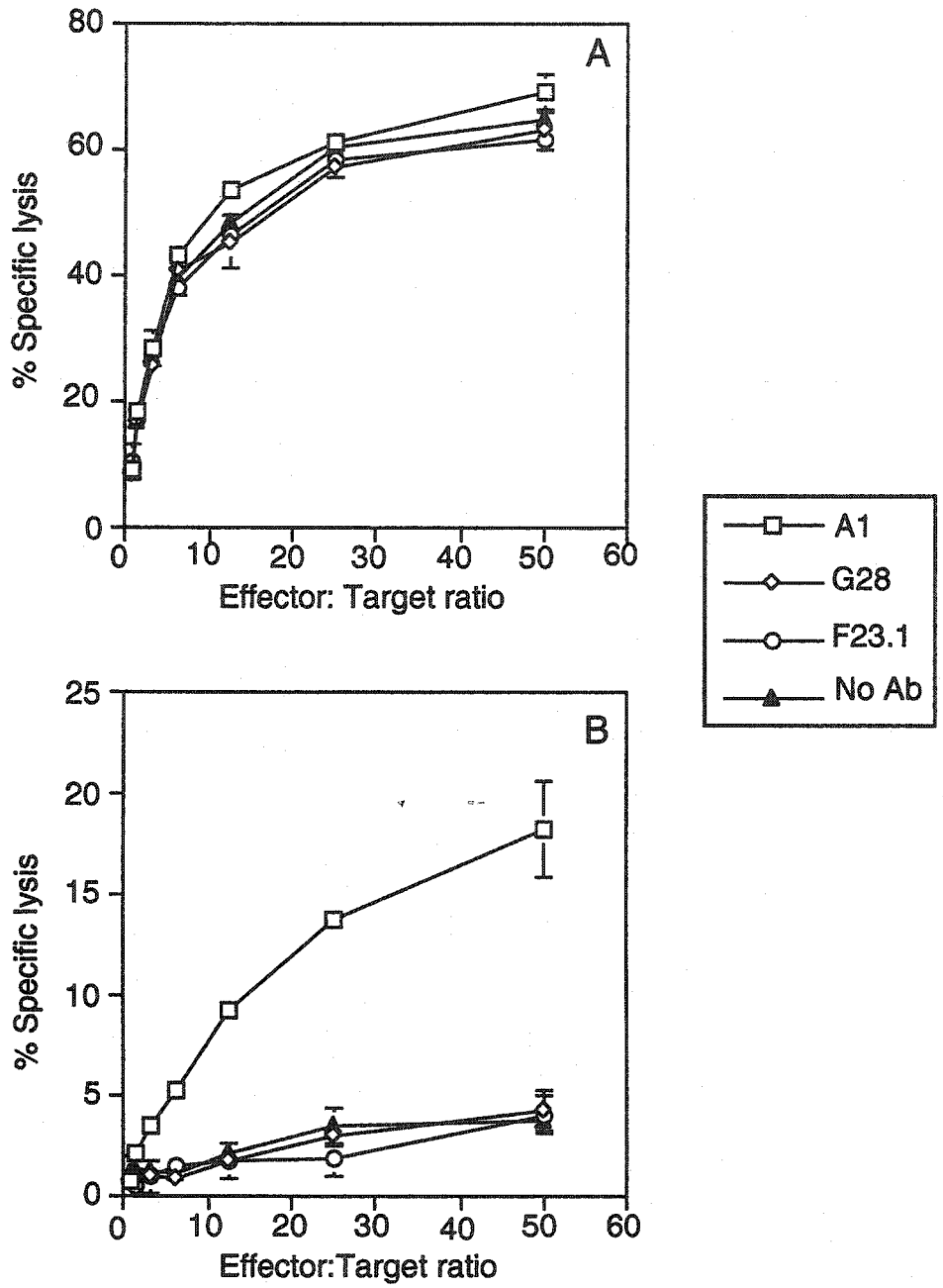

Figure 2-3. Ly-49P transfected RNK-16 mediate reverse ADCC with the A1 antibody. RNK-16 (*A*), and the 1B9 Ly-49P RNK transfectant (*B*), were incubated with 5 μg/ml of A1, G28, F23.1 or medium as indicated for 15 min prior to addition of FcR-bearing YB2.0 target cells and 4 hr cytotoxicity assay. The data represent the mean of triplicate wells ± SD.

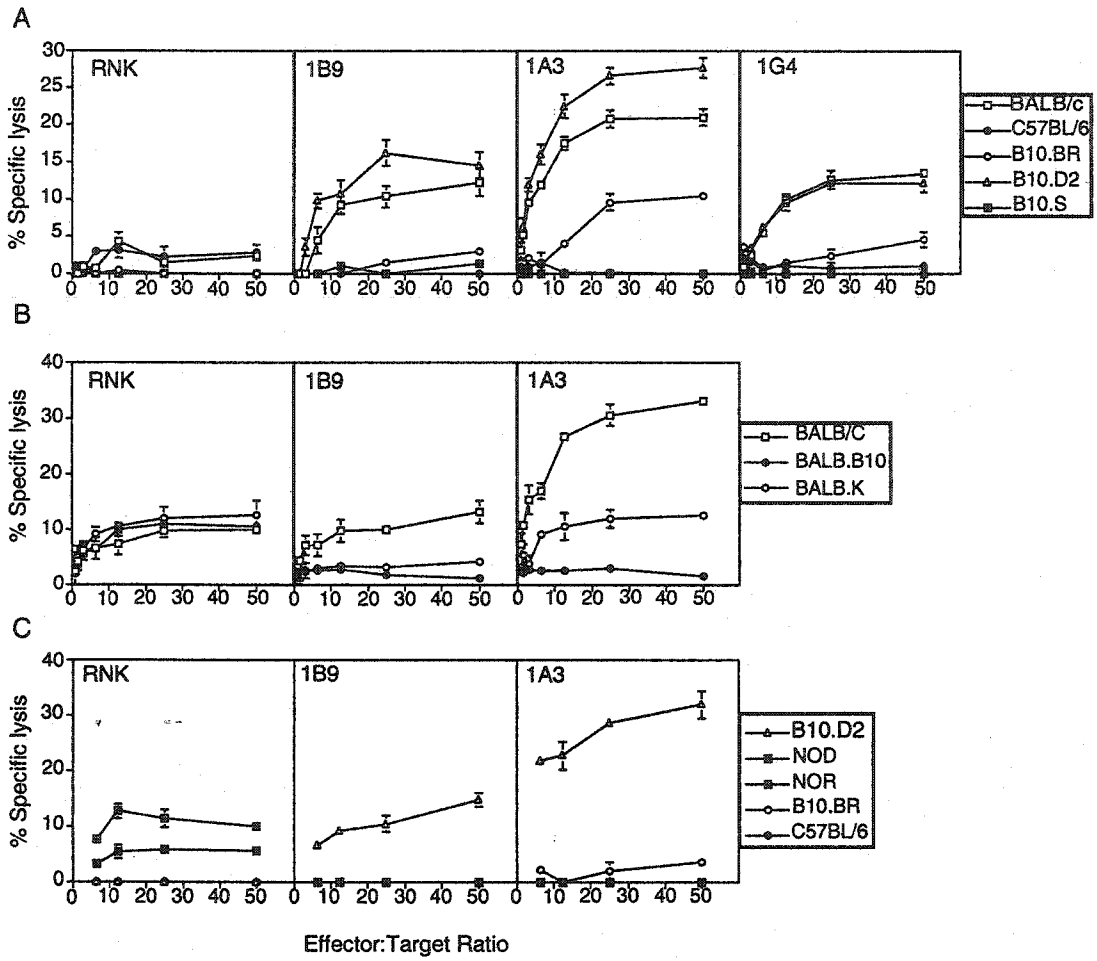

Figure 2-4. MHC dependence of Ly-49P activated RNK-16 lysis of Con A activated T cell blasts. RNK-16 cells or individual Ly-49P transfectant clones of RNK-16, 1B9, 1A3 or 1G4 were assayed for cytotoxicity against Con A blasts from various mouse strains that include H-2 congenics. Con A-activated T cell blast targets were generated from spleen cells isolated from BALB/c, C57BL/6, B10.BR, B10.D2 and B10.S (*A*), BALB/c, BALB.B10 and BALB.K (*B*) or B10.D2, NOD, NOR, B10.BR and C57BL/6 (*C*) for 4 hr cytotoxicity assays with the indicated effector cells. The data presented represent the mean of triplicate wells ± SD.

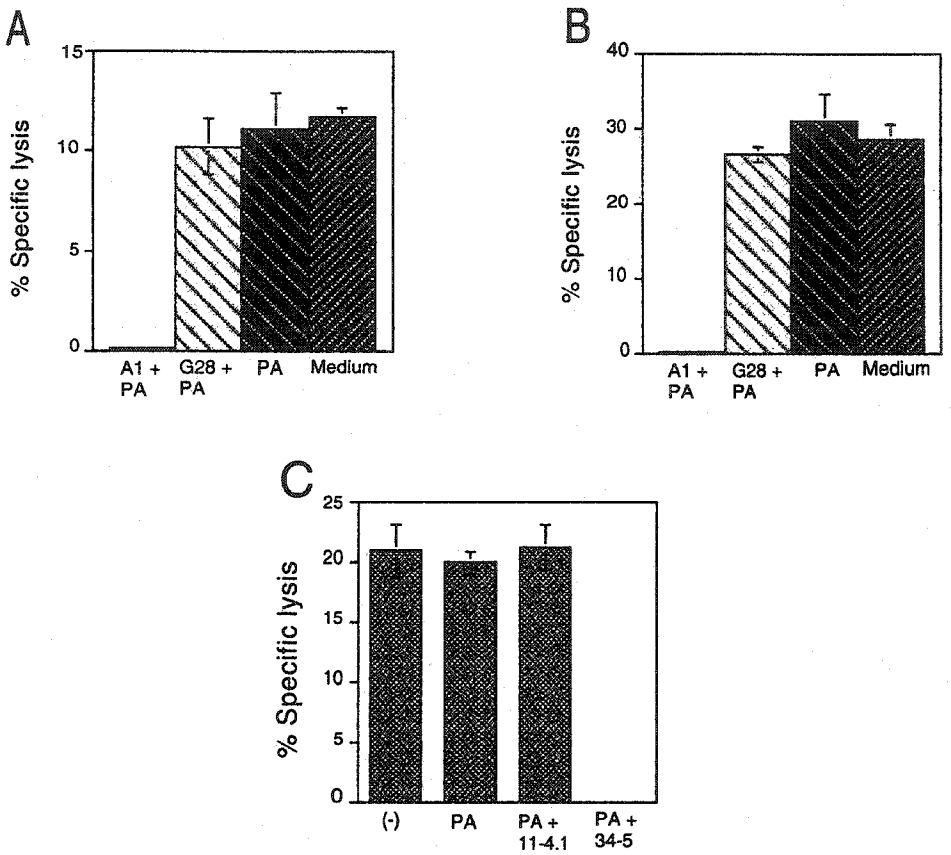

Figure 2-5. Activation of RNK-16 cytotoxicity mediated by Ly-49P recognition of Con A-activated T cell blasts is blockable by A1 and 34-5-8S antibodies. Cytotoxicity against B10.D2 Con A blast target cells by RNK-16 Ly-49P transfectant clones 1B9 (*A*), and 1A3 (*B*) was determined in the presence of medium, protein A (PA), or PA plus A1 or G28 (anti-rat CD8α) control antibody. Antibodies were preincubated with PA, 2 μg per 10 μg of mAb, for 30 min prior to addition to effector cells. Effector cells were then incubated with the mAbs and PA for 15 min prior to the cytotoxicity assay. The PA and mAbs remained in the wells throughout the cytotoxicity assay with target cells. The final concentration of antibody was 5 μg/10^6 effector cells. (*C*) Cytotoxicity against B10.D2 Con A blast targets by the RNK-16 Ly-49P transfectant 1B9 was determined in the presence of medium, PA or PA plus 34-5-8S (anti-H-2Dd α1/α2 domain) shown as 34-5, or 11-4.1(anti-H-2Kk) isotype control antibody. Antibodies were preincubated with PA as described for (*A*) and (*B*), then target cells were incubated with the mAbs and PA for 15 min prior to the cytotoxicity assay. The final concentration of antibody was 5 μg/ml. The PA and mAbs remained in the wells throughout the cytotoxicity assays. The effector to target cell ratio was 50:1 for all the assays. The 51Cr-release assays were 4 hrs. Data are means of triplicate wells ± SD.

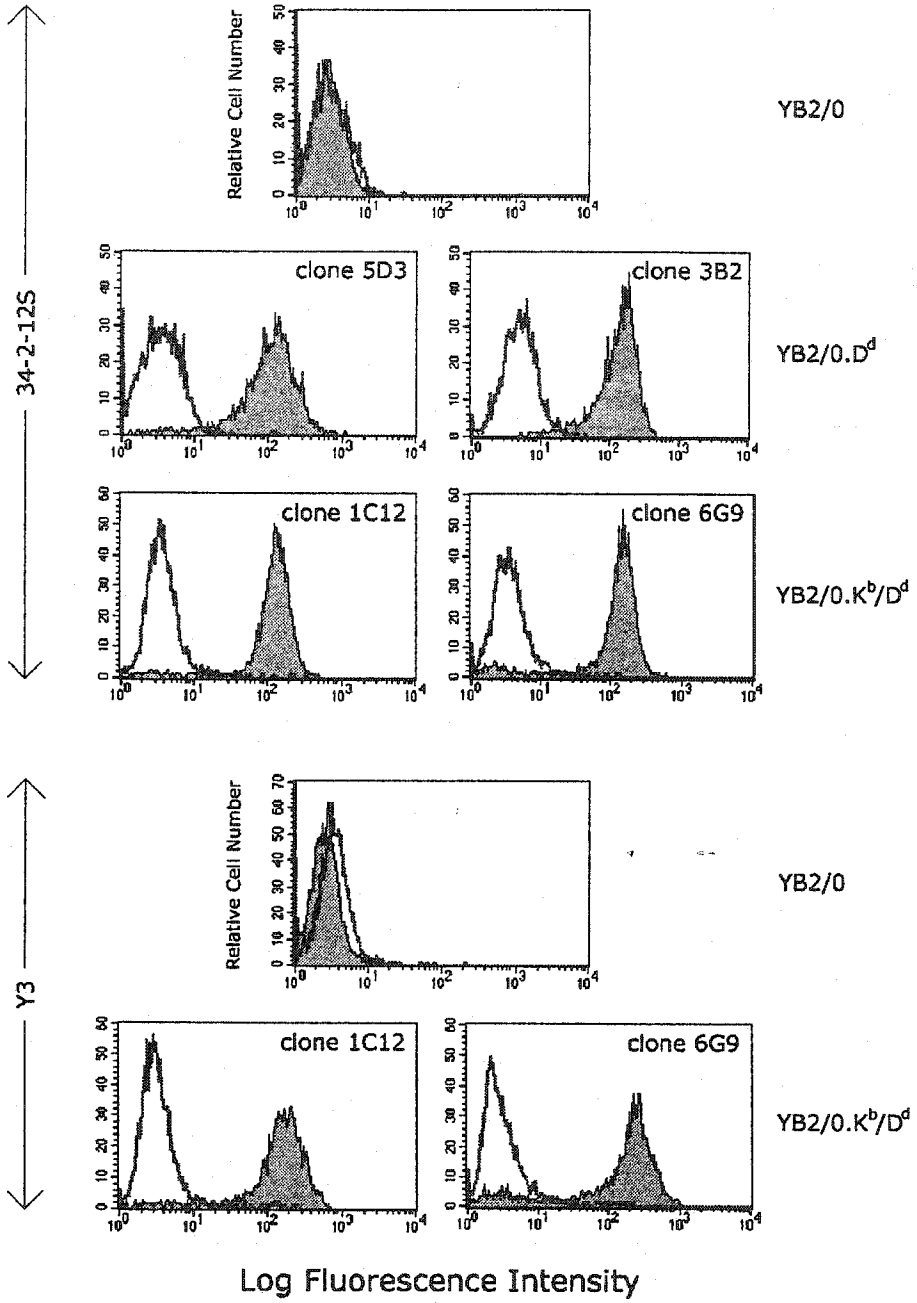

Figure 2-6. Expression of H-2D^d and an H-2K^{b}α1α2/D^{d}α3 chimera on rat YB2/0 cell transfectants. The rat myeloma YB2/0 and YB2/0 transfected with D^d, clones 5D3 and 3B2, or a chimeric K^b α1α2/D^{d}α3, clones 1C12 and 6G9, were analyzed by FACS following incubation with 34-2-12S (anti-D^{d}α3 domain, shaded) or an isotype control antibody, 14-4-4S (anti-IE^k, unshaded), and a FITC-coupled rat anti-mouse IgG (upper panels). YB2/0 and K^b/D^d chimera transfectants were also analyzed in a similar manner with the Y3 mAb (anti-H-2K^b, shaded) and an isotype control, B27M1 (anti-HLA-B27, unshaded), (lower panels).

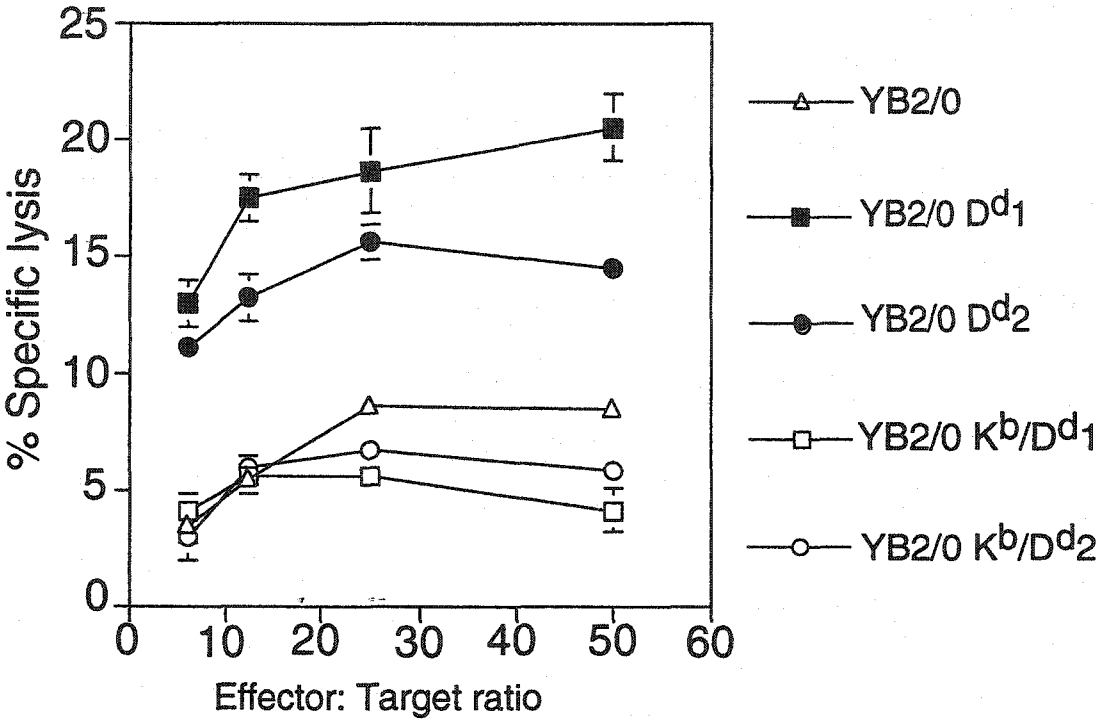

Figure 2-7. The D^d $\alpha1/\alpha2$ domain is required for RNK-16 activation through Ly49P. Lysis of YB2/0 or YB2/0 expressing H-2D^d, YB2/0.D^d1 (clone 5D3) and YB2/0.D^d2 (clone 3B2), or the chimeric $K^b\alpha1\alpha2/D^d\alpha3$, YB2/0.$K^b/D^d1$ (clone1C12) and YB2/0.K^b/D^d2 (clone 6G9) was determined in a 4 hr ^{51}Cr-release assay using the Ly49P-transfected RNK-16 cell line 1B9 as effector cells at the indicated effector to target ratios. Data are expressed as the means of triplicate wells ± SD.

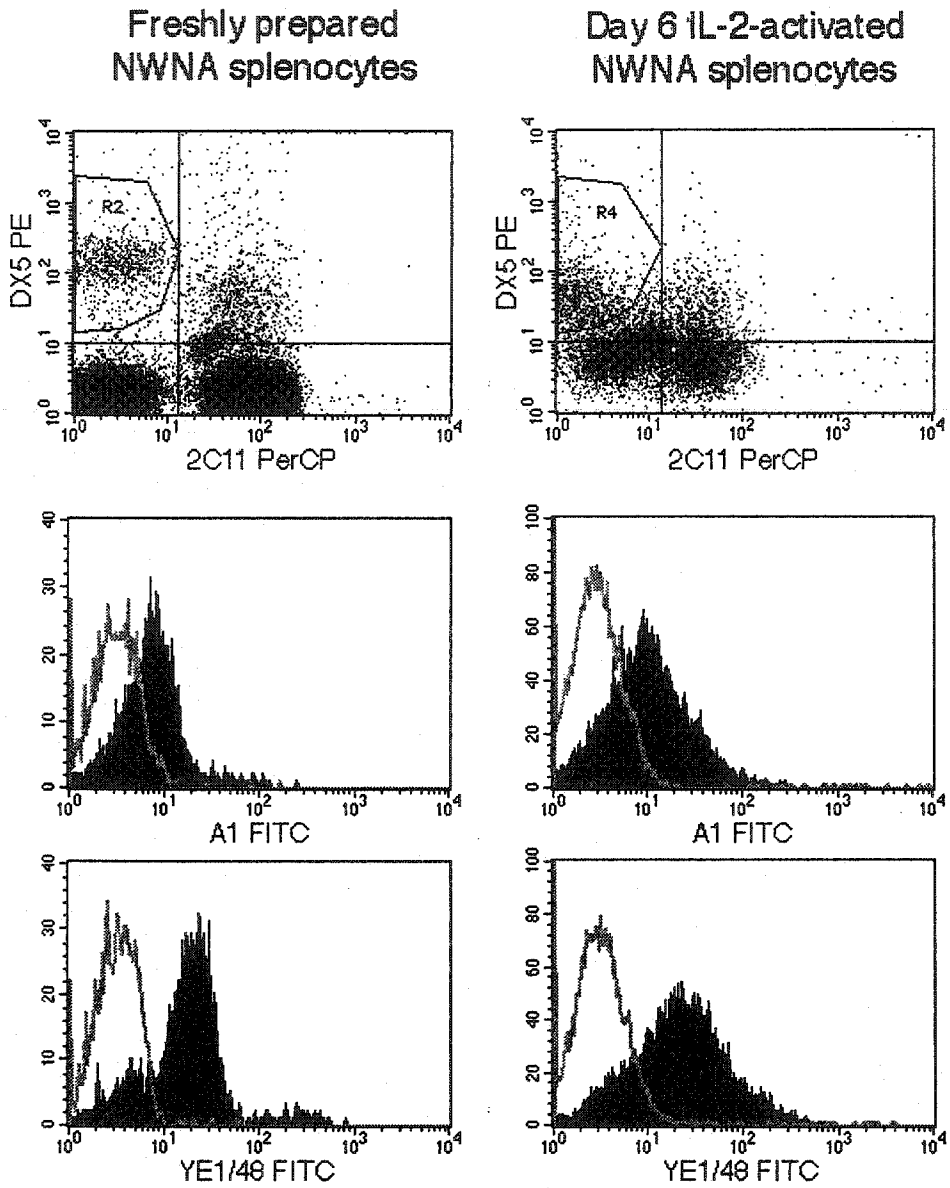

Figure 2-8. Expression of A1 and YE1/48 epitopes on freshly isolated and day six IL-2 activated $DX5^{+}CD3^{-}$ NWNA NOD spleen cells. Fresh or IL-2 activated nylon wool nonadherent (NWNA) splenocytes were prepared from five to eight week old female mice. FcR were blocked with normal mouse serum before incubating the cells with PerCP-coupled 2C11, phycoerythrin-coupled DX5 and either fluorescein-coupled A1 or YE1/48 with a fluorescein-coupled mouse anti-rat antibody. The $DX5^{+}CD3^{-}$ cells were collected in a gate and analyzed for reactivity with either A1 or YE1/48 (shaded), or the isotype control antibodies G155-178 or A23-1, respectively (unshaded). Analysis of freshly prepared NWNA splenocytes is shown on the left, and NWNA splenocytes cultured in 1000 U/ml recombinant human IL-2 for six days is shown on the right.

CHAPTER III

LY-49W, AN ACTIVATING RECEPTOR OF NONOBESE DIABETIC MICE WITH CLOSE HOMOLOGY TO THE INHIBITORY RECEPTOR LY-49G, RECOGNIZES H-2D^k AND H-2D^d [1,2]

Elizabeth T. Silver, Dong-Er Gong, Bart Hazes and Kevin P. Kane

A. Introduction

Natural killer cells function as a first line of defense to eliminate tumor cells or virally infected cells without prior sensitization (1, 2). Natural killer cells also mediate allogeneic bone marrow rejection as well as hybrid resistance, wherein an F1 mouse rejects a bone marrow transplant from a parent (3, 4). The cytotoxicity of NK cells appears to be regulated by opposing signals generated upon interaction with potential target cells. Positive signals are induced upon the interaction of activating receptor(s) with largely undefined target cell surface ligands (5-7), while NK activation is negatively

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regulated when inhibitory receptors bind class I MHC molecules on the target cell (8). Natural killer cells can lyse cells with deficient "self" class I expression, but spare cells that express normal levels of "self" class I MHC molecules. Such observations have resulted in the "missing self" hypothesis (9), which holds as one of its tenets an *in vivo* selection process in which each mature NK cell will express at least one inhibitory receptor recognizing "self" class I MHC to ensure NK cell self-tolerance (9, 10).

Natural killer cells express inhibitory receptors from the immunoglobulin and the C-type lectin superfamilies, with the human KIR and the murine Ly-49 families being the most highly characterized of the immunoglobulin and lectin-like NK inhibitory receptors, respectively (11). While KIR and Ly-49 are structurally dissimilar, they are functionally equivalent, serving as inhibitory receptors that can distinguish class I alleles in their MHC recognition. A common feature of inhibitory receptors is the presence of an ITIM (12). Engagement of the inhibitory receptor with its class I ligand on the target cell results in tyrosine phosphorylation of the ITIM (13, 14). The phosphorylated ITIM recruits the tyrosine phosphatase SHP-1, which then dephosphorylates plasma membrane proximal targets in the NK activation cascade, thus blocking NK activation (13, 14).

All NK cell inhibitory receptor families contain members that lack ITIM sequences. The ITIM-lacking molecules have a charged residue in their transmembrane domains to allow interaction with signaling adaptor molecules (12). For the KIR and Ly-49 families, this adaptor is the DAP12 signaling molecule, which contains an immunoreceptor tyrosine-based activation motif (ITAM) (11, 15-17). DAP12 interacts with the ITIM-lacking receptors via a noncovalent interaction in the transmembrane domain (11, 17). Ligation of the receptor recruits and activates Syk kinase, leading to

subsequent downstream activation events, target cell cytolysis and cytokine production (18-20). Some Ly-49 proteins can be grouped into inhibitory/activating "pairs" based on high sequence identities in their external domains (12). The functional significance of these pairs is unknown, but the considerable resemblance suggests that they recognize the same ligands. Ly-49D and Ly-49P are the only activating Ly-49 receptors for which a ligand has been determined. Ly-49D and Ly-49P recognize the H-2D^d class I MHC molecule, similar to the ITIM-containing inhibitory receptors, Ly-49A and Ly-49G (21-25).

Studies of Ly-49 receptor function have largely been confined to the C57BL/6 (B6) mouse strain. Here we examined Ly-49 gene expression and function in the non-obese diabetic (NOD) mouse, to explore strain-to-strain variation in Ly-49 expression, and because of its well-established immune dysregulation and spontaneous onset of autoimmune diabetes (26). We describe Ly-49W, a novel activating Ly-49 receptor expressed in NOD mice with high homology in its external domain to the inhibitory Ly-49G receptor. We also demonstrate that Ly-49W recognizes class I MHC molecules with strong and moderate reactivity toward H-2D^k and H-2D^d, respectively. This is the first report of an activating Ly-49 molecule recognizing an H-2^k product and provides further support for the possibility that activating Ly-49 receptors recognize class I MHC ligands.

B. Materials and Methods

Animals

Five to eight week old female AKR/J (H-2^k), CBA/J (H-2^k), BALB/c (H-2^d), DBA/2J (H-2^d), C57BL/6 (H-2^b), NOD (H-$2K^d$,D^b), NOR (H-$2K^d$,D^b), B10.BR (H-2^k), B10.D2 (B10.D2-$H2^d$/nSn; H-2^d), B10.S (H-2^s), B10 (C57BL/10; H-2^b), B10.d/b (B10.HTG-$H2^g$/2Cy; H-$2K^d$,D^b), and B10.b/d (B10.A-$H2^{i5}$H2-$T18^A$(5R)/SgSnJ; H-$2K^b$,D^d) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Additional five to eight week old NOR and NOD mice were obtained from the University of Alberta mouse breeding facility.

Hybridomas and antibodies

Hybridomas producing the following antibodies: 4D11 (rat IgG_{2a}), anti-Ly-49G (27); Cwy-3 (IgG_1), anti-Ly-49G(28); M1/42 (rat IgG_{2a}), anti-mouse class I MHC (29); 34-5-8S(IgG_{2a}), anti-H-$2D^d$ α1/α2 domain epitope (30); 34-2-12S (IgG_{2a}) anti-H-$2D^d$ α3 domain epitope (30); B8-24-3 (IgG_1), anti-H-$2K^b$ (31); B27 M1 (IgG_{2a}), anti-HLA-B27, B7 (32); and BB7.1 (IgG_1) anti-HLA-B7 (33) were obtained from ATCC (Manassas, VA), except Cwy-3 which was generated in this laboratory. Antibodies were prepared by ammonium sulfate precipitation and PBS dialysis of tissue culture supernatants obtained from hybridomas grown in protein free hybridoma medium. Purified OX-8 (IgG_1) anti-rat CD8α (34), 11-4.1 (IgG_{2a}) anti-K^k (35) and 15-5-5S (IgG_{2a}) anti-D^k (36) antibodies were purchased from BD Pharmingen (San Diego, CA). Purified rat IgG was purchased from Sigma-Aldrich (Oakville, Canada). FITC-coupled rat anti-mouse IgG, goat anti-rat

IgG, and mouse anti-rat IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cell lines

RNK-16, a spontaneous F344 rat strain NK cell leukemia cell line (37), was kindly provided by Dr. Mary Nakamura at the University of California at San Francisco. The RNK-16 cells were maintained in RPMI 1640 supplemented with 10% FCS, L-glutamine, penicillin, streptomycin and 5 x 10^{-5} M 2-mercaptoethanol (RNK medium). COS-7 SV40-transformed African green monkey kidney cells were provided by Dr. John Elliott (University of Alberta, Edmonton, Alberta, Canada). COS-7 cells were grown in Opti-MEM I medium (Life Technologies, Burlington, ON), containing 4% heat inactivated FCS (Medicorp, Montreal, Canada) and 5 x 10^{-5} M 2-mercaptoethanol.

NK cell propagation, RNA preparation, and cDNA synthesis

Interleukin-2 (IL-2)-activated NK cells were prepared from NOD, NOR or C57BL/6 strain spleen cells harvested from six- to eight-week-old female mice, as described by Smith and co-workers (38). Total cellular RNA was isolated with TRIzol reagent (Life Technologies), and reverse-transcribed using SuperScript II (Life Technologies) with an oligo-dT primer.

Cloning of NOD Ly-49 transcripts

Ly-49 transcripts were amplified with Advantage cDNA Polymerase (Clontech, Palo Alto, CA) using the sense cloning primer 5'-CCCAAGATGAGTGAGCAGGAGG and the antisense cloning primer 5'-GAGAGTCAATGAGGGAATTTATCC. The PCR

products were purified using QIAquick spin columns (Qiagen Inc., Santa Clarita, CA) and directly ligated into the TA cloning vector p3T (Mo Bi Tec, Göttingen, Germany). The individual cDNA clones were divided into two groups: ITIM-encoding clones which could be PCR-amplified using the antisense cloning primer and the sense primer 5'-GGAGACTCAAGGGCCCAGAG, or ITIM-lacking clones which could be PCR-amplified by using the antisense cloning primer and the sense primer 5'-CTRRAAAAGCTGGCCTCAGAGTY (where R=A/G and Y=C/T). To verify the nucleotide sequences of the ends of the open reading frames and to obtain sequence of the non-coding gene-flanking regions for cloning, we performed 5'-rapid amplification of cDNA ends (5'-RACE) of *Ly-49W*. Poly A^+ RNA was isolated from total RNA, obtained from IL-2-activated NK cells using Oligotex spin columns (Qiagen), and used to synthesize a cDNA library with the Marathon cDNA Amplification Kit (Clontech). The gene-specific primer used for *Ly-49W* 5'-RACE was 5'-GCCTGGCCTACACTCTATAGATGTAGAAC. To obtain full-length cDNA clones, we used the following primers: *Ly-49G*, 5'-ATTTTAACTGAGAACA TACTTCATACATCAT (sense) and 5'-GATTGTCTCTCTCTTTGCACTTTTACA (antisense); *Ly-49W*, 5'-TTTAAAAGAGAACATACTCTACATCCTC (sense) and the same antisense primer as *Ly-49G*. Sequencing reactions were performed using a dideoxy terminator method and analyzed on an ABI 373A automated sequencer (Applied Biosystems, Foster City, CA).

Transfection and flow cytometric analysis

COS-7 cells: DNA containing the coding regions of *Ly-49* cDNAs was inserted into the *XbaI/EcoR*I sites of the mammalian expression vector pCI-neo (Promega, Madison, WI).

Sequence encoding the mature mouse DAP12 protein was inserted into the pFLAG-CMV-1 expression vector (Sigma-Aldrich, Oakville, Canada) to create an epitope-tagged chimeric protein. Vectors were then transfected into COS cells using LipofectAMINE (Life Technologies). Approximately 48 hours after transfection, the cells were incubated with the monoclonal antibodies 4D11 or Cwy-3, or isotype control antibodies M1/42 or B8-24-3, respectively. Secondary antibodies, either FITC-labeled goat anti-rat IgG or rat anti-mouse IgG, respectively, were subsequently added for an additional incubation, whereupon the samples were analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

RNK cells: The cDNA encoding Ly-49W was inserted into the XhoI/XbaI sites of the bicistronic vector BSRαEN (generously provided by Dr. Andrey Shaw, Washington University, St. Louis, MO) and transfected into RNK-16 cells using the protocol described by Nakamura et al.(14). In brief, four million cells were transfected with 20 μg of plasmid linearized with SacI by electroporation at 200 mV and 960 μFD. Transfected cells were cloned in 96-well microtiter plates in complete RNK medium supplemented with 1 mg/ml G418 for drug selection. Expression of Ly-49W on transfectant clones was measured by FACS analysis with the 4D11 antibody after blocking FcR with normal mouse serum. Reactivity of transfectant clones with Cwy-3 and OX-8 antibodies was also determined after blocking with purified rat IgG. The BB7.1 antibody was used as an isotype control. Primary antibody binding was detected with mouse anti-rat and rat anti-mouse fluorescein-coupled antibodies, respectively, using a FACScan flow cytometer.

Generation of Con A T cell blast target cells

Con A-activated T cell blasts were prepared from spleen cells of various mouse strains. Fifteen million spleen cells were cultured at $5.0x10^6$ cells/ml in RPMI 1640 with 10% heat-inactivated FCS, 2-mercaptoethanol and 3.0 μg/ml Con A (Sigma-Aldrich) for 48 hr. Blast cells were recovered after three washes in RPMI 1640 medium.

Cytotoxicity assays

Target cells were labeled at 37°C with 100 to 150 μCi of $Na^{51}CrO_4$ (^{51}Cr) (Mandel/NEN Life Science Products, Guelph, Canada), for 1 hr if tumor cells, or 1.5 hr if Con A blast cells. Following extensive washes, $1x10^4$ ^{51}Cr-labeled target cells were incubated for 4-5 hr at 37°C in V-bottomed microtiter plates with RNK-16 cells or RNK-16 cells transfected with Ly-49W at various effector to target ratios in triplicate. After the incubation, plates were centrifuged for 5 min and 100 μl of supernatant was removed and counted in a gamma counter. The percent specific lysis was determined as [(experimental release - spontaneous release)/(maximum release - spontaneous release)] x 100. To perform the reverse antibody dependent cellular cytotoxicity (rADCC) experiments, untransfected RNK-16 cells and the Ly-49W transfectants of RNK-16 were preincubated for 15 min with 20 μg/ml of the Cwy-3 or OX-8 antibodies or medium alone prior to addition of FcR-expressing YB2/0 target cells and subsequent 4 hr cytotoxic assay as described. For antibody inhibition experiments, antibodies were incubated with soluble protein A (PA) [(2 μg / 10 μg of mAb), (Sigma-Aldrich)] or a mix of protein A and protein G (PG) [(4 μg/10 μg of mAb) ICN Pharmaceuticals, Costa Mesa, CA)] for 30 min prior to addition to effector cells or target cells. Effector cells or target cells were

preincubated with the mAb and PA for 15 min prior to the cytotoxicity assay. In the case of two antibodies being employed simultaneously in the same wells in receptor blocking studies, each antibody is used at the indicated concentrations in the figure. The mAbs and PA or PA/PG were present throughout the cytotoxicity assays. All cytotoxicity assays were repeated a minimum of three times.

C. Results

Cloning of noninhibitory Ly-49 members with homology to Ly-49G

Recent studies suggest that Ly-49 gene expression varies between mouse strains (25, 39, 40). Furthermore, the complex hybridization patterns in Southern blots of various mouse strains suggest that not all Ly-49 family members have been identified (41). Indeed, investigation of Ly-49 expression in mouse strains such as 129J and CBA/J, have resulted in the identification of novel activating Ly-49 molecules (40, 42). Here we examine Ly-49 expression in the non-obese diabetic (NOD) mouse strain, an animal model of insulin-dependent diabetes mellitis (IDDM). Non-obese diabetes resistant (NOR) strain mice are identical to NOD at most, but not all, genetic loci and do not develop diabetes. We designed an RT-PCR strategy to clone cDNAs encoding both activating and inhibitory Ly-49 family members from IL-2-activated NK cells of NOD and NOR strain mice. From this, we obtained cDNAs encoding both ITIM-containing and ITIM-lacking receptors.

The ITIM-containing cDNAs prepared from NOD IL-2 activated NK cells include a novel allele of the inhibitory *Ly-49G* gene with a nucleotide identity of 98.8% and 98.9%, and an amino acid identity of 97.0% and 98.1% compared to the C57BL/6 and

BALB/c alleles, respectively (Fig. 3-1A). The NOD *Ly-49G* allele more closely resembles both the C57BL/6 and BALB/c alleles than they resemble each other (97.9% nucleotide and 95.9% amino acid identities). The NOD transcripts described in this comparison correspond to *Ly-49G2*, first defined in the C57BL/6(B6) strain (Fig. 3-1A). NOR NK cells also express *Ly-49G2* (GenBank accession number AF074457) with 100% sequence identity to NOD *Ly-49G2*. Similar to the B6 strain, NOD NK cells also express alternatively spliced RNA transcripts encoding a larger *Ly-49G* form, *Ly-49G1*^{NOD}, in which there is an extension of 13 amino acid residues in the extracellular membrane-proximal stalk domain of the receptor (GenBank accession number AF283248). In addition, NOD NK cells express *Ly-49G* transcripts that, through alternative splicing, lack exon 3 encoding the transmembrane segment and a portion of the cytoplasmic domain (GenBank accession number AF283253).

The ITIM-lacking NOD IL-2 activated NK cDNAs include sequences of two novel *Ly-49* transcripts. Both sequences show strong similarity with two genomic fragments from B6 mice that were originally designated *Ly49m* (43). To determine the relationship of our new sequences with *Ly-49m* from the B6 strain, we cloned and sequenced the full *Ly-49m* transcript from B6 mouse cDNA (Fig. 3-1B). This showed that *Ly-49m* is not functionally expressed in B6 mice as there is a premature stop codon near the predicted beginning of exon 4 (Fig. 3-1B). The B6 *Ly-49m* sequence differs by only 10 bases and 8 amino acids from one of our new sequences, corresponding to 98.7% and 97.0% sequence identity on the DNA and protein levels, respectively. This extent of sequence identity is comparable to that found between alleles of *Ly-49G*. Thus, we are tentatively designating this NOD Ly-49 product as the NOD allele of *Ly-49m*. Interestingly, the NOD gene does not contain the premature stop codon suggesting that it

represents a functional receptor. This is an additional example of variation in Ly-49 expression between different mouse strains.

Our other new NOD Ly-49 transcript also resembles *Ly-49m* with 18 bases and 15 amino acids substituted, corresponding to 97.7% and 94.3% sequence identity on the DNA and protein level, respectively. However, this product must represent a distinct gene since we have already shown above that NOD encodes an even more closely related *Ly-49m* homologue. Hence, we have designated this NOD Ly-49 transcript *Ly-49W* (Fig. 3-1A). There are two distinct *Ly-49W* mRNA transcripts which result from alternative splicing at the beginning of exon 3. We have previously demonstrated that this form of alternative splicing also occurs in *Ly-49D* and *Ly-49H* (44). Splice variants containing the coding sequence for cytoplasmic residues Val-Cys-Ser are named *Ly-49D1* and *Ly-49H1*, while those lacking this coding sequence are termed *Ly-49D2* and *Ly-49H2*. Hence, we are using the designations *Ly-49W1* and *Ly-49W2* for transcripts that encode or lack the Val-Cys-Ser sequence, respectively (Fig. 3-1B). We have also found both splice variants with identical sequences in the NOR mouse strain (GenBank accession numbers AF074459 and AF074463).

Recently the complete sequence of a novel receptor, Ly-49L, was determined. Ly-49L is found in CBA/J, C3H (42) and BALB/c mice (Fig. 3-1B), and its nucleotide and amino acid sequence does not differ in these strains. Ly-49L has 94.3% amino acid sequence identity with Ly-49W (Fig. 3-1B), the same level of divergence as observed between Ly-49W and Ly-49M. However, Ly-49W and Ly-49L amino acid sequences are identical in exons 2 to 5, with all substitutions taking place in the C-terminal region encoded by exons 6 and 7 (Fig. 3-1B, and data not shown). The genetic relationship of Ly-49W and Ly-49L is unclear. The overall level of amino acid identity strongly suggests

that Ly-49L and Ly-49W are separate genes. It remains a possibility, however, that Ly-49W and Ly-49L are alleles of the same gene, but a substitition of exons 6 and 7 from another gene has occurred in Ly-49L or Ly-49W. Detailed genomic analysis of Ly-49 genes in multiple mouse strains will be necessary to address these possibilities. In any event, the amino acid sequence of the external domain of Ly-49W is closer to that of Ly-49G than those of Ly-49M or Ly-49L.

The carbohydrate recognition domain (CRD) sequence of Ly-49W (exons 5 to 7) is strikingly similar to the same region of the inhibitory receptor Ly-49G, with an amino acid sequence identity of 97.6% compared to the Ly-49G alleles of both the B6 and NOD strains (Fig. 3-1A). In the case of the stalk region, these numbers are 83.8% and 82.4%, respectively. Interestingly, the cytoplasmic/transmembrane domain is rather different as it lacks the ITIM motif and contains an arginine in the middle of its transmembrane domain (Fig. 3-1). This is the hallmark of the known activating receptors Ly-49D, Ly-49H, and Ly-49P (17, 25, 40). Below, we will present evidence that Ly-49W is indeed an activating receptor. Accordingly, Ly-49G/Ly-49W form an inhibitory/activating pair with opposite regulatory effects, but closely related CRDs. This is analogous to other Ly-49 inhibitory/activating pairs that have previously been reported (17, 25, 40, 41). The studies described below will examine the function and specificity of Ly-49W.

Ly-49G-reactive antibodies 4D11 and Cwy-3 recognize NOD Ly-49W

Serological determinants may be shared between NOD Ly-49G and Ly-49W due to the high degree of amino acid identity in their external domains. To address this possibility, we transiently expressed cDNAs encoding these molecules in COS cells and performed FACS analysis with antibodies reactive with Ly-49G2. The 4D11 antibody

was initially demonstrated to detect an epitope expressed on several mouse strains (27) and subsequently was determined to recognize Ly-49G (23). The Cwy-3 mAb has been shown to have a much more restricted specificity by recognizing Ly-49G of C57BL/6, but not several other strains (28). Cwy-3 and 4D11 recognize Ly-49G of NOD origin, as indicated by substantial staining of COS cell transfectants with these antibodies (Fig. 3-2, upper panels). Thus, Cwy-3 recognizes the NOD and B6 alleles of Ly-49G. Ly-49W expression on COS cells is not readily detected with Cwy-3 or 4D11 antibodies, unless Ly-49W is cotransfected with DAP12 (Fig. 3-2, lower panels). Thus, Ly-49W is recognized by the Ly-49G-"specific" mAbs 4D11 and Cwy-3, but, similar to other noninhibitory Ly-49 family members (17, 40), appears to require the accessory molecule DAP12 for efficient cell surface expression.

Ly-49W mediates reverse ADCC

The noninhibitory Ly-49D, Ly-49H and Ly-49P receptors have been shown to be activating receptors by their ability to mediate reverse antibody dependent cell-mediated cytotoxicity (rADCC), or redirected lysis, against FcR-expressing targets (25, 45, 46). Since the antibodies that bind Ly-49W also recognize the inhibitory receptor Ly-49G, the experimental system we chose (14), employed the RNK-16 NK cell line transfected with mouse Ly-49W as effector cells. In this way, we could be certain there was no antibody cross-reactivity and the effector cells would be Ly-49W$^+$, yet Ly-49G$^-$. Transfected RNK-16 cells were screened by FACS analysis to select for clones stably expressing Ly-49W. A number of transfectant clones expressing Ly-49W were obtained as determined by acquisition of reactivity with the 4D11 and Cwy-3 antibodies and three clones were selected for study (Fig. 3-3). Cotransfection of mouse DAP12 was not required for

expression of Ly-49W on RNK-16 cells as is observed with Ly-49D, another noninhibitory Ly-49 (24), presumably because rat DAP-12 substitutes for the mouse accessory protein. Expression of rat CD8, a constitutively expressed endogenous rat receptor, was retained following transfection with Ly-49W (Fig. 3-3).

RNK-16 cells can efficiently lyse the FcR-expressing target cell line YB2/0 [(14), Fig. 3-4A)], but there is often a marked decrease in target cell killing when the RNK-16 cells are transfected with an activating receptor, such as Ly-49D (24). This effect may be due to a competition for, and sequestration of, a limiting activation component, possibly DAP12, by the transfected activating receptor, relative to an activating receptor that is normally involved in YB2/0 recognition. Such a substantial reduction of direct YB2/0 cytolysis is seen with two Ly-49W transfectant clones 2C4 and 10G5 (Fig. 3-4B and C). Lysis of FcR expressing YB2/0 target cells by the Ly-49W transfected RNK-16 clones 2C4 and 10G5 could be substantially increased when Ly-49W on the effector cells was cross-linked by the Cwy-3 antibody (Fig. 3-4B and C). In contrast, cross-linking of effector cell CD8α by the OX-8 antibody did not increase target cell lysis, although there is comparable cell surface expression of CD8α and Ly-49W on each of the transfected RNK clones tested (Fig. 3-3). These results indicate that Ly-49W can mediate rADCC and can be considered an activating receptor. Since high baseline levels of YB2/0 lysis appear to be retained for the Ly-49W transfectant clone 7E8, perhaps due to a greater abundance of signaling molecules, little augmentation of cytolysis by Cwy-3 cross-linking of Ly-49W expressed on 7E8 can be detected with this clone (Fig. 3-4D). Untransfected RNK-16-mediated lysis of YB2/0 cells was unaffected by the Cwy-3 and OX-8 antibodies (Fig. 3-4A).

The cytolytic activity of Ly-49W-transfected RNK-16 cells is MHC dependent

The H-2D^d class I MHC molecule is a ligand for some inhibitory Ly-49 receptors, including Ly-49G (23) and the activating receptor Ly-49D (24). To test whether class I MHC molecules, especially of the H-2^d haplotype, are recognized by Ly-49W, we examined Ly-49W-mediated killing of Con A-activated T cell blast target cells prepared from splenocytes of mouse strains expressing different MHC haplotypes. None of the Ly-49W transfectant clones lysed B6 Con A targets (H-2^b), whereas all of the transfectants lysed Con A blasts from the AKR and CBA mouse strains expressing H-2^k MHC molecules (Fig. 3-5A). The B10.D2 and DBA/2 mouse strains bearing H-2^d MHC molecules were lysed at a very low level except in the case of the highly lytic clone 7E8, which kills H-2^d Con A blasts at a moderate level (Fig. 3-5A). No lysis of any of the Con A T cell blast targets was observed with the untransfected RNK-16 cells. Together, these results suggest that Ly-49W may recognize H-2^k, and possibly H-2^d, product(s).

To further define the importance of MHC molecules in Ly-49W recognition, we examined the lysis of Con A blasts generated from congenic B10 mouse strains that differ only in their MHC genes, by the Ly-49W transfectants. Striking differences were observed in the lysis of the Con A blasts generated from the B10 congenic mice. The B10.BR (H-2^k) Con A blasts were readily lysed by all three Ly-49W transfectant clones (Fig. 3-5B). The B10.D2 (H-2^d) Con A blasts were lysed at low to moderate levels, whereas no detectable lysis of B10.S (H-2^s) Con A blasts was observed (Fig. 3-5B). These results suggest that Ly-49W is an activating receptor that recognizes one or more H-2^k and H-2^d product(s).

Ly-49W recognizes H-2D^k and H-2D^d

The previous results suggest that there is an H-2^k and perhaps an H-2^d product or products recognized by Ly-49W. To determine which H-2^k class I product(s) is recognized by Ly-49W, we attempted to block Ly-49W-mediated lysis of B10.BR (H-2^k) Con A blasts with antibodies that recognize either H-2K^k or H-2D^k. Soluble Protein A (PA) was used to bind the antibody Fc domain to prevent ADCC by the FcR-expressing RNK-16 cells. Neither the 11-4.1 antibody, which recognizes H-2K^k (35), nor the isotype control antibody B27 M1 block lysis of B10.BR Con A blasts (Fig. 3-6). In contrast, the 15-5-5S antibody, which recognizes H-2D^k (36), blocks lysis of B10.BR Con A blasts by both Ly-49W-transfectant clones 7E8 and 10G5 in a dose dependent manner (Fig. 3-6). These results indicate that Ly-49W recognizes H-2D^k.

Con A blast lysis results using congenic mice suggest that Ly-49W has a low to moderate level of recognition of an H-2^d molecule. The Ly-49W transfectant 7E8 lyses B10.D2 Con A blasts to a moderate level and was therefore used in further experiments to determine which H-2^d product can be recognized by Ly-49W. We took two complementary approaches to address this issue. First, 7E8 lysis of Con A blasts generated from intra-MHC recombinant mouse strains B10.b/d (H-2K^b, H-2D^d) and B10.d/b (H-2K^d, H-2D^b) was compared with the B10 (H-2^b) negative control. Con A-activated T cell blasts from the intra-MHC recombinant which expresses H-2D^d were lysed, whereas those from the recombinant expressing H-2K^d and the B10 strain were not (Fig. 3-7A). Second, we attempted to block lysis of B10.D2 Con A blasts by the 7E8 Ly-49W transfectant clone with two antibodies that recognize H-2D^d: 34-5-8S which recognizes the D^d α1/α2 domains, and 34-2-12S which recognizes the D^d α3 domain. Both D^d-specific antibodies blocked Ly-49W-mediated RNK lysis of the B10.D2 blasts

(Fig. 3-7B), whereas the isotype control antibody or Protein A had no effect. The 34-5-8S antibody blocking was more effective than 34-2-12S through the titration of these antibodies, consistent with previous studies of Ly-49A interactions with H-2D^d (22). This suggests that Ly-49W interacts with H-2D^d in the α1/α2 domains, as does Ly-49A. In addition, since the 34-2-12S antibody also blocks Ly-49W interaction with D^d, albeit less efficiently, the α3 domain may also contribute to the interaction, or this antibody may sterically hinder Ly-49W interaction with the D^d α1/α2 domains. In any case, these results indicate that Ly-49W recognizes H-2D^d.

Lysis of H-2^k-expressing targets by Ly-49W transfected RNK cells is inhibited by antibodies that recognize Ly-49W

The foregoing experiments demonstrated that Ly-49W-transfected RNK cells readily recognize H-2D^k expressing targets, while untransfected RNK cells do not recognize them at all. To confirm that the observed lytic activity is mediated by Ly-49W, we examined whether antibodies recognizing Ly-49W could block the cytolytic activity. Both of the Ly-49W-recognizing antibodies 4D11 and Cwy-3 reduced the killing of B10.BR Con A blasts by clone 10G5 (Fig. 3-8). The Cwy-3 antibody inhibited the majority of the lysis observed with the Ly-49W transfectant clone 10G5 and it was more effective than 4D11 (Fig. 3-8). Combining 4D11 and Cwy-3 antibodies did not increase inhibition of lysis. Similar results were obtained with other Ly-49W transfectants (data not shown). These results support the role of Ly-49W in mediating RNK lysis of H-2D^k target cells.

Ly-49W^{NOD} transfectants of RNK-16 cells do not lyse NOD or NOR Con A blasts

The NOD and NOR mouse strains express H-2K^{d} and H-2D^{b} MHC molecules. Our previous results indicated that neither of these MHC proteins is recognized by Ly-49W (Fig. 3-7A). To confirm that Ly-49W from NOD and NOR mice does not recognize "self" MHC, we compared lysis of Con A blasts generated from NOD, NOR, AKR and B6 mice by Ly-49W RNK transfectants. As in previous experiments, AKR Con A blasts served as very good targets, while B6 blasts were not lysed by the Ly-49W expressing RNK-16 effector cells (Fig. 3-9). In comparison, we found that NOD or NOR Con A blasts were not lysed by clones 2C4 and 10G5, and lysed to only a very limited extent, if at all, by the more highly cytolytic clone 7E8 (Fig. 3-9). These results demonstrate that Ly-49W from NOD and NOR mice does not recognize self antigens. We conclude from our studies that Ly-49W of NOD/NOR mice is an activating receptor specific for allogeneic class I MHC molecules.

D. Discussion

The B6 strain has served as a prototype for the study of Ly-49 gene expression and function. However, apparent strain-specific transcripts encoding novel activating receptors have recently been described: Ly-49P from the 129/J and NOD mouse strains (25, 40), and Ly-49L from CBA/J, C3H and BALB/c mice (42) (Fig. 3-1B). Viable transcripts for none of these receptors have been found in the B6 mouse. We demonstrate in this report that NOD and NOR mice express two new mRNA transcripts encoding activating Ly-49 receptors. For one of these, Ly-49W, we could not find an allele in B6 mice. The other, Ly-49M, shares 98.7% nucleotide sequence identity with a transcript that we cloned from B6 mice and which incorporates the sequence of two genomic

fragments previously designated Ly-49M. Interestingly, NOD Ly-49M transcripts appear to be functional, while B6 Ly-49M transcripts contain a premature stop codon. These findings present clear evidence that Ly-49 repertoires differ significantly between mouse strains. We further show that in at least one case, Ly-49M, this difference is due to a gene defect rather than to different levels of gene transcription.

Ly-49L, M and W are closely related genes as indicated by sequence comparisons, suggesting that they result from relatively recent gene duplication and perhaps exon exchange. The ability of Ly-49 genes to undergo duplication and genetic recombination is similar to the functionally equivalent KIR receptors in primates, perhaps in response to similar evolutionary pressures (47-50). Evidence of human KIR gene recombination involving genes widely separated in the genome (47, 48), supports a contribution of gene conversion or nonhomologous recombination in the generation of receptor diversity. There are specific areas within the KIR gene complex where these variations occur, suggesting that "hot spots" of gene duplication in the KIR gene complex may exist (48). The Ly-49 gene complex may have similar variability in different mouse strains as the results in this report and others suggest (25, 40, 42). The relatively recent formation of Ly-49L, M, and W by gene duplication and exon exchange events suggest that these genes may be located near a similar "hot spot" in the NK gene complex (NKC) region (51), encoding Ly-49 genes. Thus, gene duplication and nonhomologous recombination or gene conversion may contribute to the generation of polymorphism in both Ly-49 and KIR gene families.

In addition to substantial homology between Ly-49W, Ly-49M and Ly-49L, there is a striking similarity of the Ly-49W carbohydrate recognition domain (CRD) with that of the Ly-49G inhibitory receptor. For exons 5 to 7, encoding the CRD, the amino acid

sequence identity between Ly-49W and G is 97.6%. The sequence conservation drops to 83.8% for exon 4, which encodes the less well conserved, stalk region. For exons 2 and 3 the amino acid sequence identity is only 58.2% and, unlike Ly-49W and M, Ly-49G contains the ITIM motif in its cytoplasmic domain. Based on these results, Ly-49G and Ly-49W and M should be considered as inhibitory/activating pairs of Ly-49 receptors, similar to other examples such as: Ly-49A and Ly-49P (25, 40); Ly-49O and Ly-49D (40); Ly-49C/Ly-49I/Ly-49J and Ly-49H (41); NKG2-A and NKG2-C/NKG2-E (52, 53); NKR-P1B and NKR-P1A/NKR-P1C (54). The generation of inhibiting/activating receptor gene "pairs" may involve non-homologous gene recombination events or gene conversion.

Ly-49W joins Ly-49D and Ly-49P as activating Ly-49 receptors which recognize class I MHC ligands (24, 25). Our findings with Ly-49W strengthen the conclusion that activating Ly-49 receptors, like inhibitory Ly-49 receptors recognize class I MHC ligands. We have demonstrated that Ly-49W is the first activating Ly-49 receptor to recognize an H-2^k product. Ligands for Ly-49W were determined to be H-$2D^k$ and, with weaker interaction, H-$2D^d$. This novel recognition pattern contrasts with Ly-49D and Ly-49P which only react significantly with H-$2D^d$ (24, 25). From NK lytic assays using Con A blast target cells, it was determined that the inhibitory Ly-49G receptor of B6 mice recognizes D^d, D^r and an undefined H-2^k product (55). This work is contradicted by the finding that Ly-49G does not bind soluble H-$2D^k$ tetramers and does not mediate binding to H-2^k bearing cells in cell-cell adhesion assays (56). However, the high sequence identity between the CRD of Ly-49G and Ly-49W and our finding that Ly-49W binds H-$2D^k$, suggest that Ly-49G should be re-examined for H-$2D^k$ reactivity.

With the availability of the Ly-49A/H-2D^d complex co-crystal structure (57), it is now possible to correlate sequence variation among Ly-49 and class I MHC molecules with their known interaction specificities. Ly-49A has a known binding specificity for H-2D^d with only weak binding to H-2D^k (22, 56). Conversely, we have demonstrated here that Ly-49W reacts strongly to D^k and only weakly to D^d. Among the D^d residues that interact directly with Ly-49A in the crystal structure, only two are not conserved in D^k (Lys173-Asn174 in D^d and Glu173-Leu174 in D^k). Similarly, among the Ly-49A residues that interact directly with D^d in the crystal structure, five positions are not conserved in Ly-49W, all in the hexapeptide 244-249 (NCDQVF in Ly-49A and DCGKSY in Ly-49W). Molecular modeling suggests that compensating changes within these sequences confer the differential class I MHC specificities/affinities of Ly-49A and Ly-49W (not shown).

Self tolerance of NK cells is believed to be maintained by expression and function of inhibitory receptors specific for self class I MHC proteins (10). Activating receptors may react with self MHC as well, and tolerance could be maintained by coexpression of inhibitory receptors that dominantly suppress activating receptor signals. For instance, it has been reported that tolerance of Ly-49D$^+$ NK cells of self cells expressing H-2D^d is likely maintained by the coexpression of Ly-49G and other Ly-49 inhibitory receptors (55). We demonstrate in this report that Ly-49W does not interact with self class I MHC proteins of the NOD/NOR mouse strains, but is instead alloreactive. For this reason, self tolerance in NOD/NOR strains is not likely to be affected by Ly-49W. It remains to be determined whether, and if so how, Ly-49W$^+$ NK cells are tolerant in F1 animals such as CBAxNOD, where an identified Ly-49W ligand is expressed.

We found that the Cwy-3 and 4D11 antibodies were both reactive with Ly-49W, an activating receptor, and there were differences between the results obtained using Cwy-3 and 4D11. While rADCC using RNK-16 transfectants of Ly-49W could be demonstrated with the Cwy-3 antibody, the 4D11 antibody was unable to mediate rADCC (data not shown). Additionally, the Cwy-3 antibody was somewhat better at blocking Ly-49W mediated lysis of H-2^k Con A blasts than 4D11 (Fig. 3-8). Differences in antibody affinity and/or site of attachment are most likely the cause of these different results. Our results suggest that it cannot be assumed that the inhibitory Ly-49G receptor is what is recognized by 4D11 in every mouse strain, since in addition, or instead, it may recognize an activating Ly-49 such as M, L, W or related receptors in certain strains.

The existence of activating receptors that recognize class I MHC molecules is not readily predicted by the "missing self hypothesis", which prompts the question: what is the role of MHC-specific activators in NK cell function? Activating Ly-49 members may function in conjunction with inhibitory receptors by recruiting kinases to phosphorylate ITIM sequences following ligand binding, thus augmenting the recruitment of SHP-1 and thereby the function of coexpressed inhibitory receptors. This possibility remains to be explored. The expression of inhibitory Ly-49 receptors occurs through a stochastic process that obeys a simple statistical product rule for expression of multiple Ly-49 receptors (10). In contrast, a recent report provides evidence to suggest that there is non-stochastic expression of two Ly-49 activators, D and H, in that there is a greater tendency for them to be coexpressed on NK cells (46). These observations suggest that Ly-49 activators may coordinate their activities and possibly function independent of inhibitory Ly-49 receptors in some NK cells (46). However, this analysis involved only two Ly-49 activating receptors and it is now clear that several more exist. Further studies that

include additional activating Ly-49 receptors will be necessary before a paradigm can be established for the function of this form of Ly-49 receptor. Identifying the extent and diversity of the Ly-49 family, both activating and inhibitory members, as well as their expression patterns and ligand specificities, should provide an opportunity to fully understand Ly-49 receptor functions. Identification and characterization of Ly-49W, a class I MHC-specific activating receptor, contributes to this goal.

E. Acknowledgements

We thank Dr. Mary Nakamura (UCSF) for RNK-16 cells and for helpful advice in transfecting them, Dr. Andrey Shaw (Wash. Univ. St. Louis) for the BSRαEN expression vector. We also thank Dr. John Elliott (Univ. of Alberta) for provision of NOD and NOR mice and COS-7 cells.

F. Author Contribution to Data

Elizabeth Silver generated the C57BL/6, BALB/c, NOD and NOR cDNA libraries, cloned by RT-PCR the *Ly-49* transcripts described in this study, performed the COS transient transfections, generated the stable Ly-49W transfectant clones, and did the flow cytometric analyses for this study. Dong-Er Gong performed the cytotoxicity assays published in this study. Bart Hazes did the molecular modeling referred to in the discussion.

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A

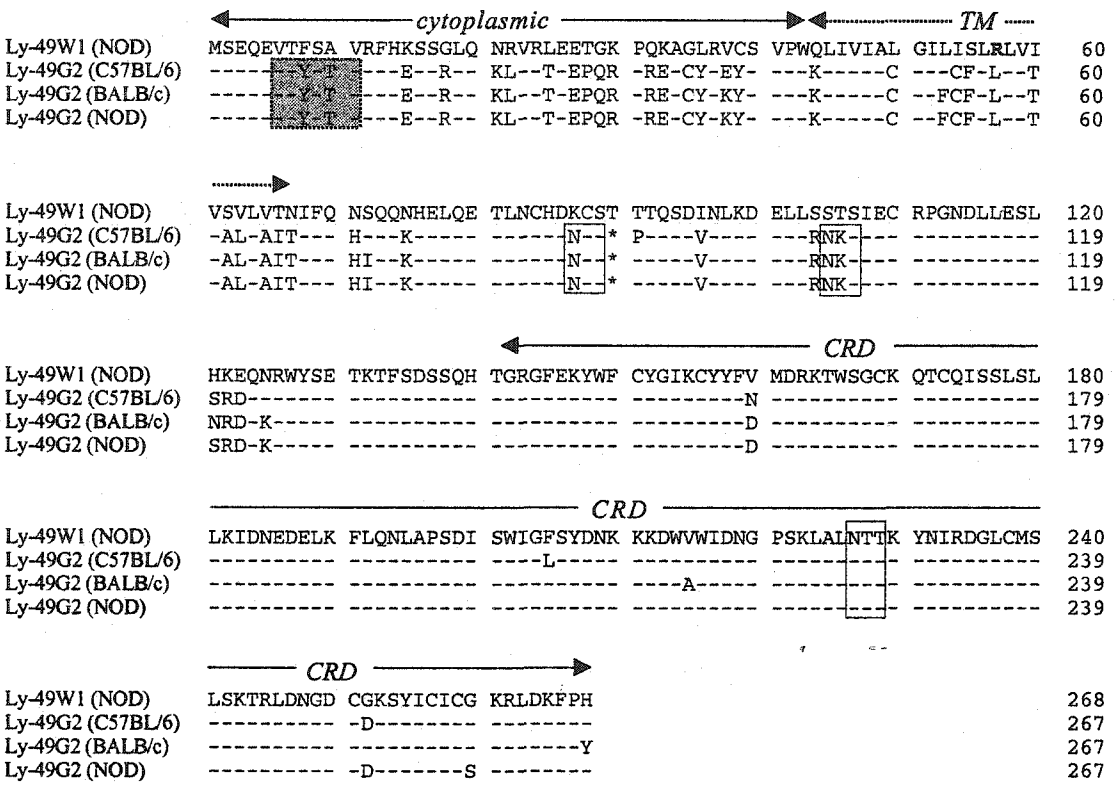

Figure 3-1A. Comparison of the predicted amino acid sequences of Ly-49W with Ly-49G, Ly-49L and Ly-49M. *A*, The predicted amino acid sequence of Ly-49W is aligned with those of the Ly-49G alleles from the C57BL/6, BALB/c, and NOD mouse strains. Dashes indicate sequence identity, asterisks indicate sequence gaps, and potential N-linked glycosylation sites are boxed (unshaded). Predicted protein domains are indicated by horizontal arrows above the sequences. The ITIM sequence is shaded and boxed with dotted lines. Nucleotide sequences are available under the GenBank accession numbers: AF283250 (Ly-49W1^{NOD}), AF307946 (Ly-49G2$^{BALB/c}$), and AF283249 (Ly-49G2^{NOD}).

B

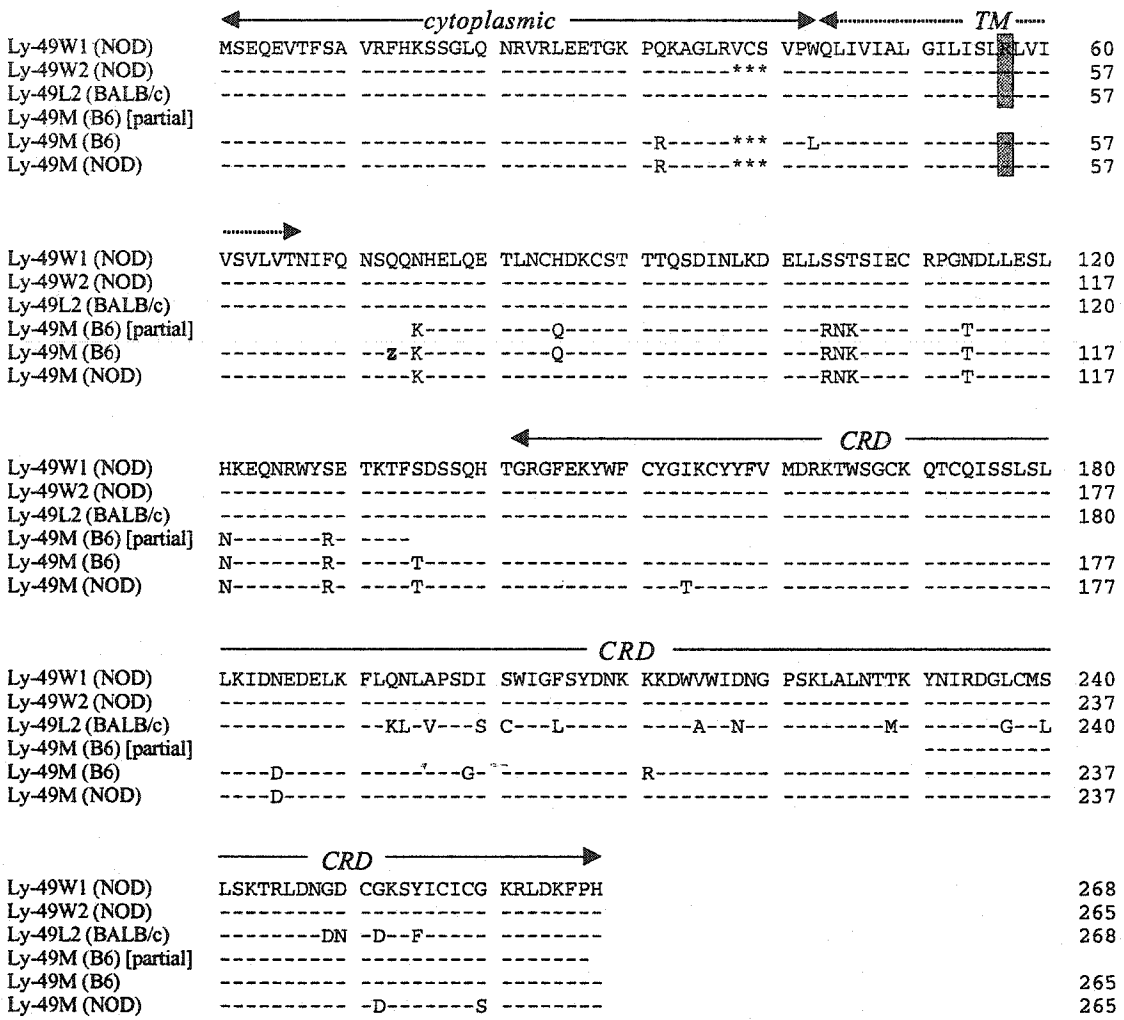

Figure 3-1B. Comparison of the predicted amino acid sequences of Ly-49W with Ly-49G, Ly-49L and Ly-49M. *B*, The alignment of the predicted protein sequences of splice variants Ly-49W1 and Ly-49W2 with Ly-49L2 of BALB/c mice as well as Ly-49M from C57BL/6 and NOD mouse strains. Dashes indicate sequence identity and asterisks indicate sequence gaps. Predicted protein domains are indicated by horizontal arrows above the sequences. The arginine in the transmembrane region is shaded and boxed with solid lines. The termination codon is indicated by a lower case bold z. Nucleotide sequences are available under the GenBank accession numbers: AF283250 (Ly-49W1^{NOD}), AF283251 (Ly-49W2^{NOD}), AF307947 (Ly-49L$^{BALB/c}$), AF028133 (Ly-49M^{B6} [partial, exons 4 and 7]), AF074460 (Ly-49M^{B6}) and AF283252 (Ly-49M^{NOD}).

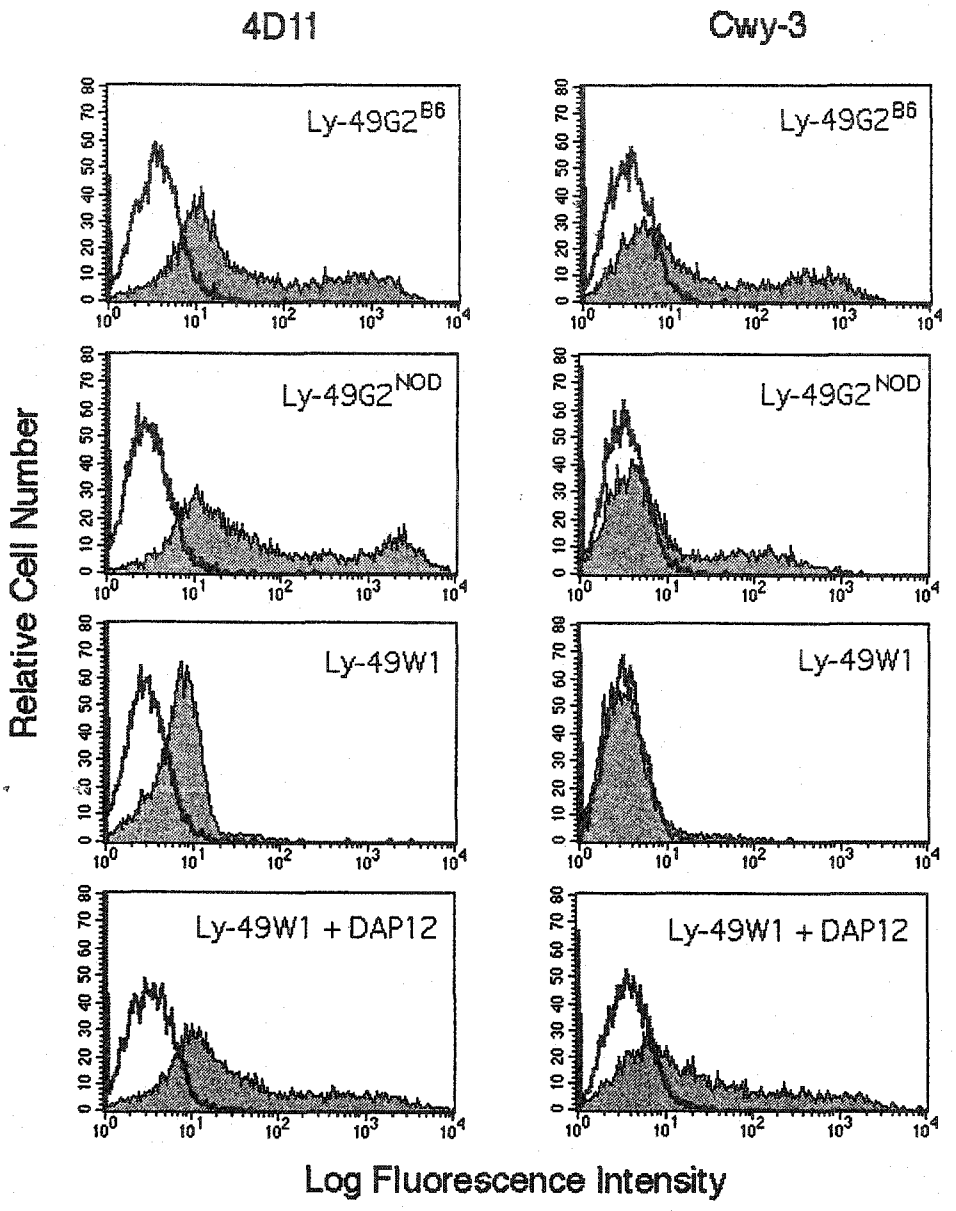

FIGURE 3-2. Ly-49W is recognized by the 4D11 and Cwy-3 antibodies. COS-7 cells transfected with Ly-49G from C57BL/6 (B6) or NOD mice, or Ly-49W alone or Ly-49W cotransfected with murine DAP12, were analyzed by flow cytometry using the antibodies 4D11 or Cwy-3 (shaded), or the isotype control antibodies M1/42 or B8-24-3 (unshaded), respectively.

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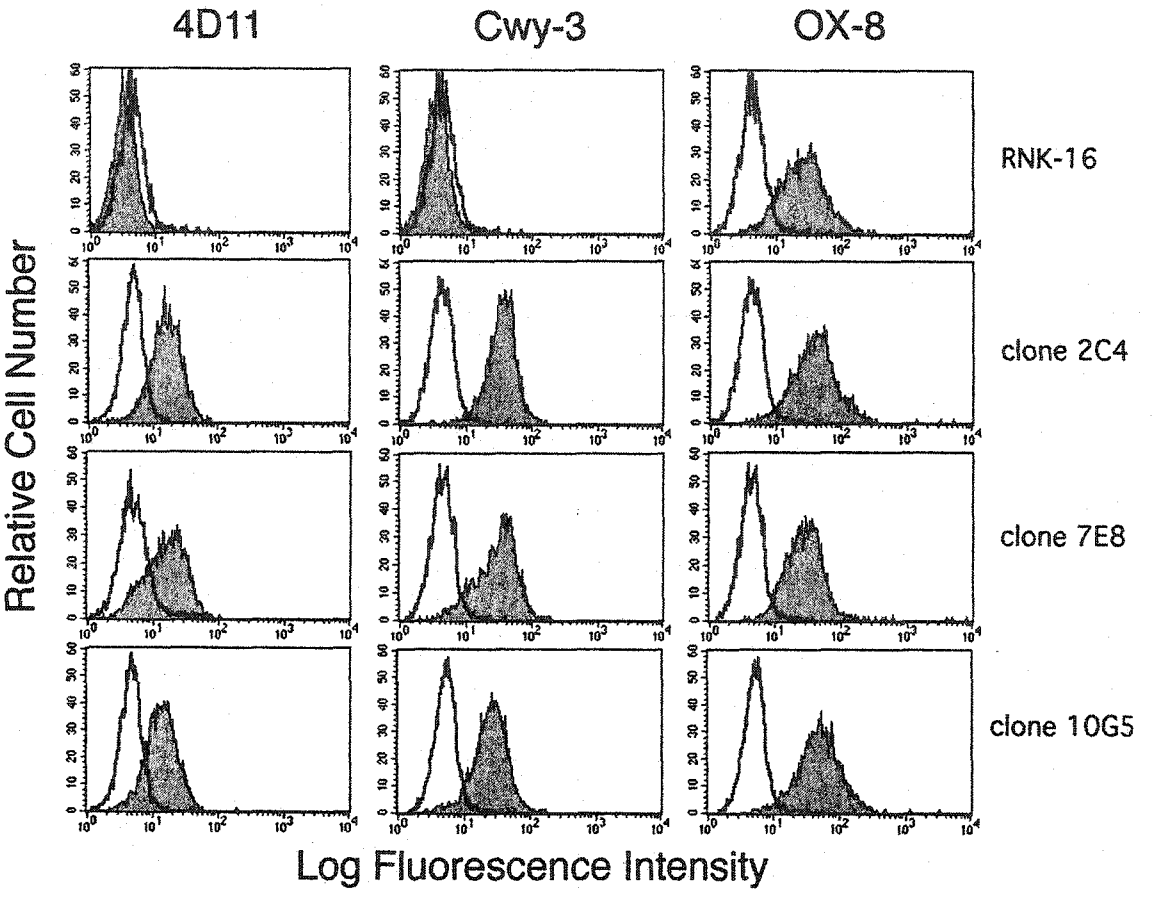

FIGURE 3-3. Expression of Ly-49W on RNK-16 transfectants. FACS analysis of Ly-49W-transfected RNK-16 clones 2C4, 7E8, and 10G5 with the antibodies 4D11 or Cwy-3 (shaded), or no first antibody, or BB7.1 isotype control (unshaded), respectively. Expression of rat CD8α on RNK-16 cells was analyzed with the OX-8 antibody (shaded) or the isotype control BB7.1 (unshaded). Antibody binding was detected with fluorescein-coupled mouse anti-rat or rat anti-mouse Abs.ly. Lysis of DBA/2 Con A blasts by RNK.WG^{B6} clone 1E12, or CBA Con A blasts by RNK.WG$^{BALB/c}$ clone 1G9, was measured with no Ab or in the presence of anti-Ly-49G Ab (Cwy-3 or 4D11, respectively) or control Abs (OX-8 or Y13-238, respectively), at the indicated concentrations. Antibodies were preincubated with 1 μg protein A/protein G (PA/PG) per μg Ab for 30 min before they were added to effector cells for 15 min. Subsequent cytotoxicity was determined at an E/T ratio of 12.5:1 in 4-h ^{51}Cr-release assays. Data represent the mean of triplicate wells ± SD.

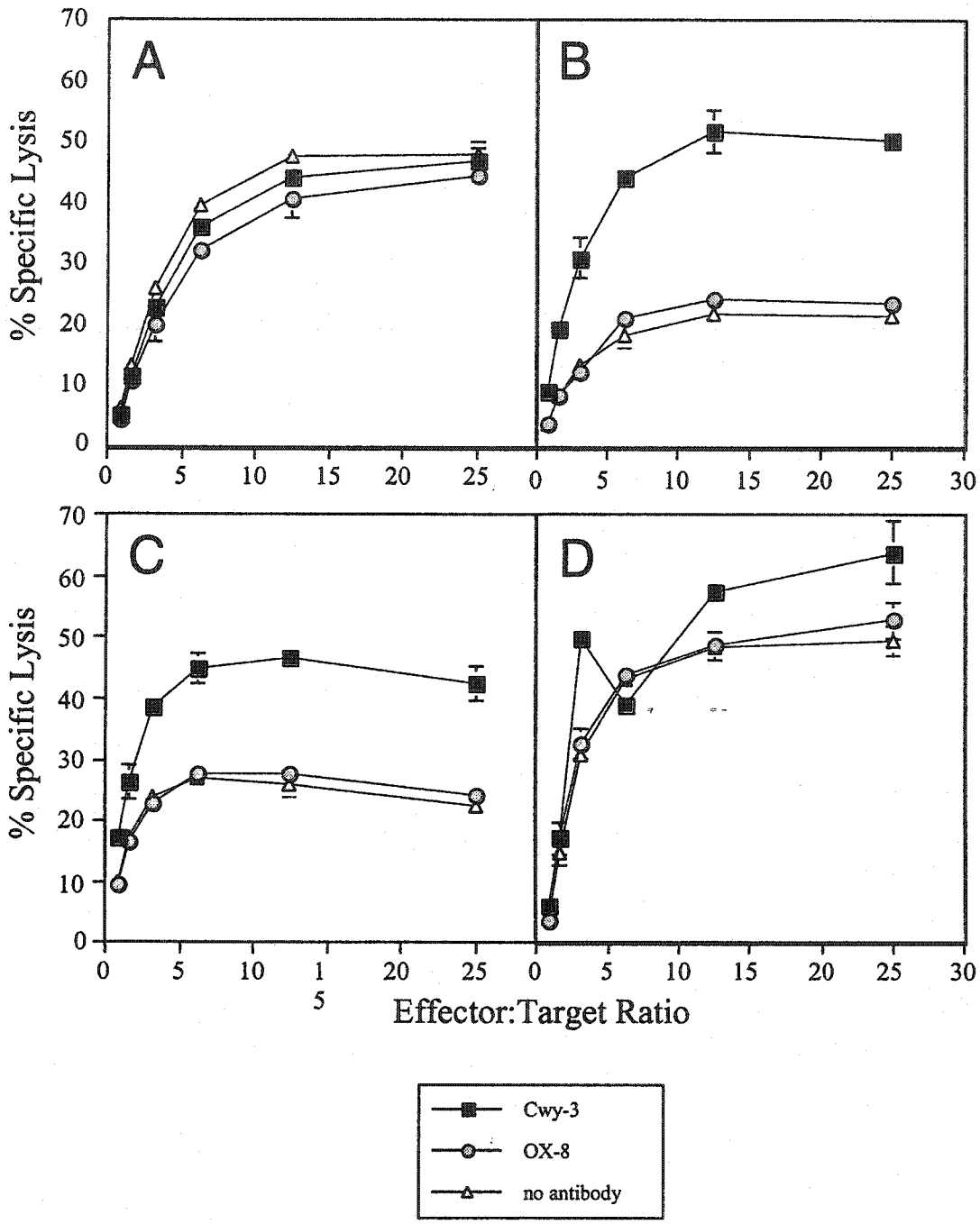

Figure 3-4. Ly-49W expressed on RNK-16 cells mediates reverse ADCC with the Cwy-3 antibody. RNK-16 cells (*A*) and the Ly-49W transfectant clones 2C4 (*B*), 10G5 (*C*) and 7E8 (*D*) were incubated with the indicated antibodies at 20 mg/ml or with medium for 15 min prior to addition of FcR-bearing YB2/0 target cells and a 4-h cytotoxicity assay. The data represent the mean of triplicate wells +/- SD.

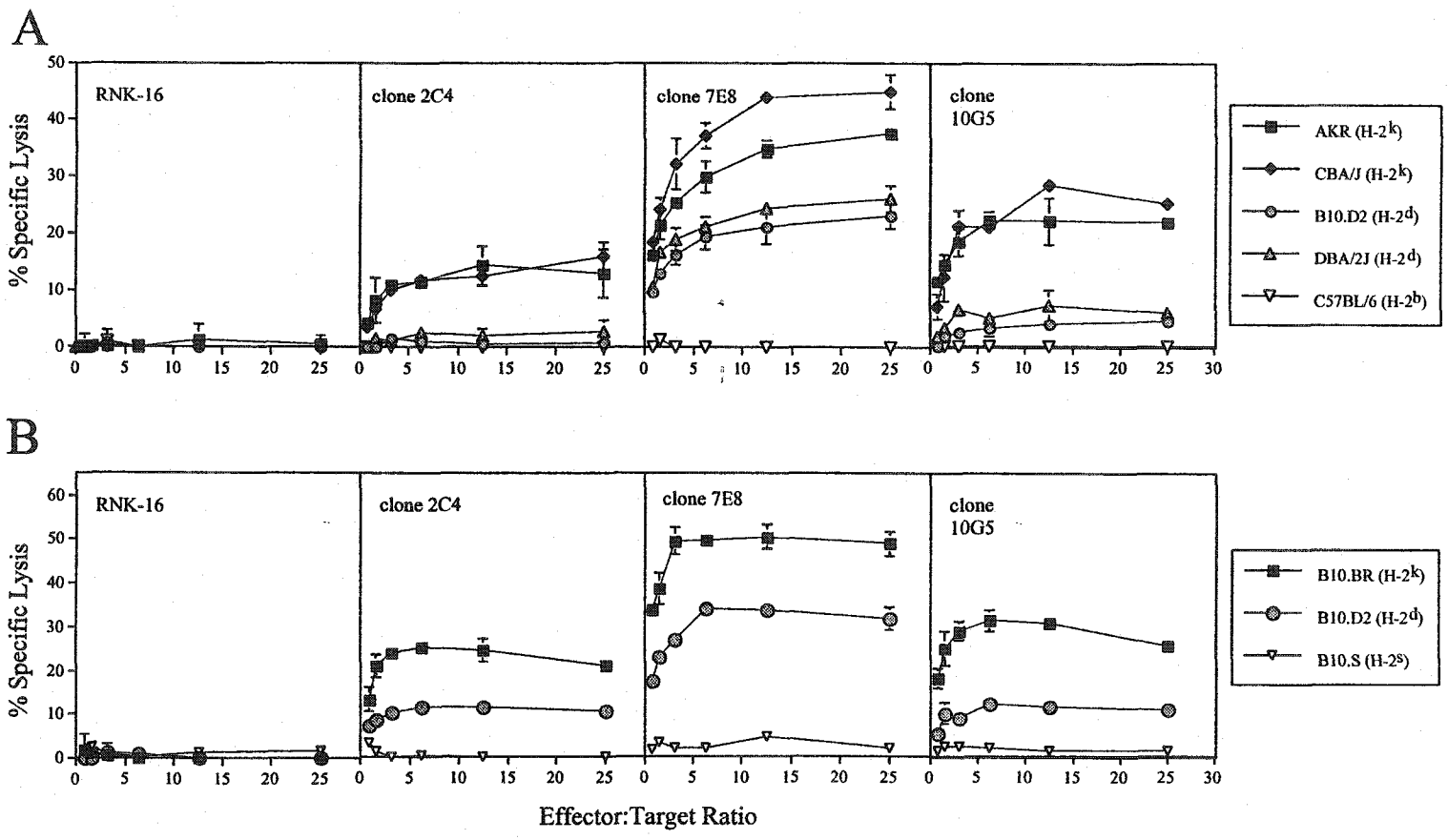

Figure 3-5. Ly-49W activation of RNK-16-mediated lysis of Con A blasts is MHC dependent. RNK-16 and Ly49W-transfected RNK-16 cells were tested as effectors in the lysis of Con A-activated T cell blast targets generated from splenocytes isolated from various mouse strains: *A*, H-2^k (AKR, CBA/J), H-2^d (B10.D2, DBA/2J), and H-2^b (C57BL/6); *B*, B10 congenic targets expressing H-2^k (B10.BR), H-2^d (B10.D2) and H-2^s (B10.S). Effector to target cell ratios are indicated and cytotoxicity was measured after four hours. The data represent the mean of triplicate wells $\pm$ SD.

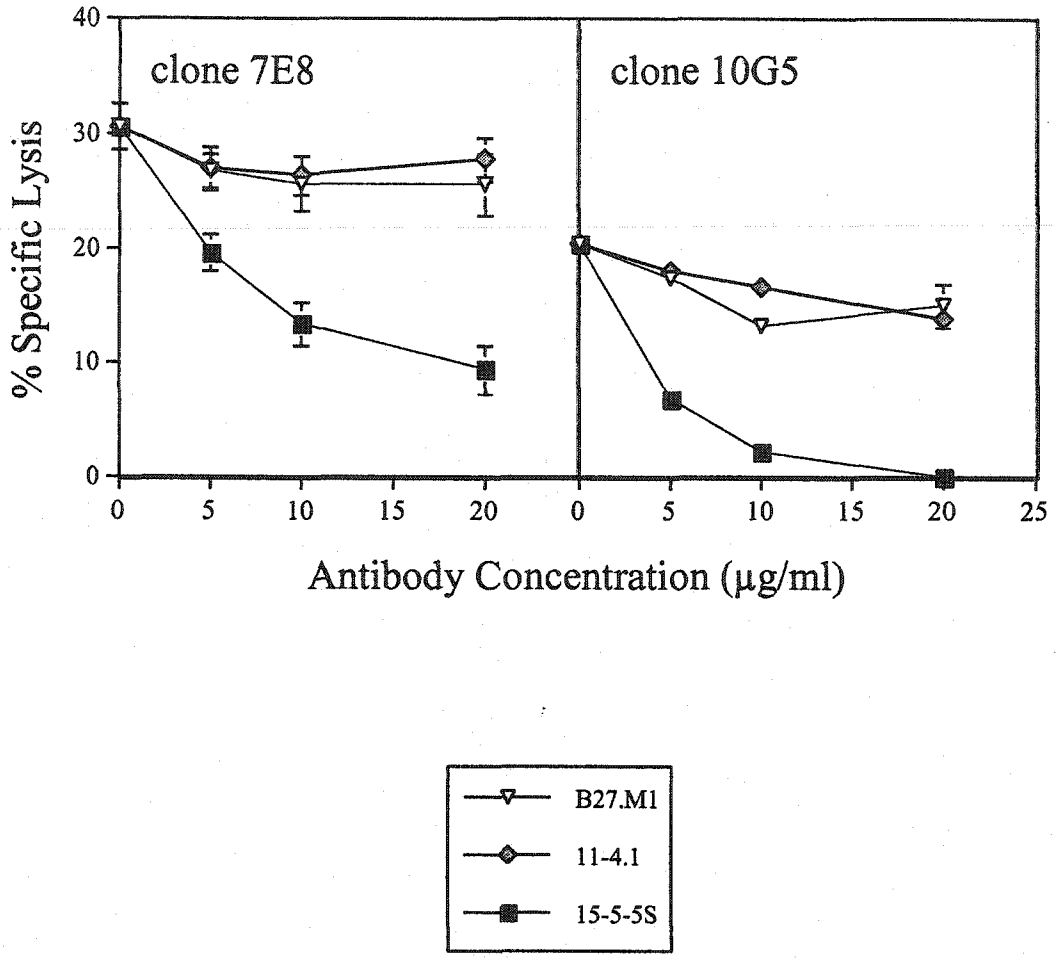

Figure 3-6. Ly-49W recognizes H-2D^k. Lysis of B10.BR (H-2^k) Con A blasts by the 7E8 and 10G5 Ly-49W transfectants was determined in the presence of PA and various concentrations of 11-4.1 (anti-K^k), 15-5-5S (anti-D^k) or B27 M1 (isotype control) antibodies. Antibodies were preincubated with PA (2 mg/10mg of mAb) to prevent ADCC. Effector cells were added to provide a 12.5:1 effector:target cell ratio. Cytotoxicity was measured after 4 h. Data are the means of triplicate wells ± SD.

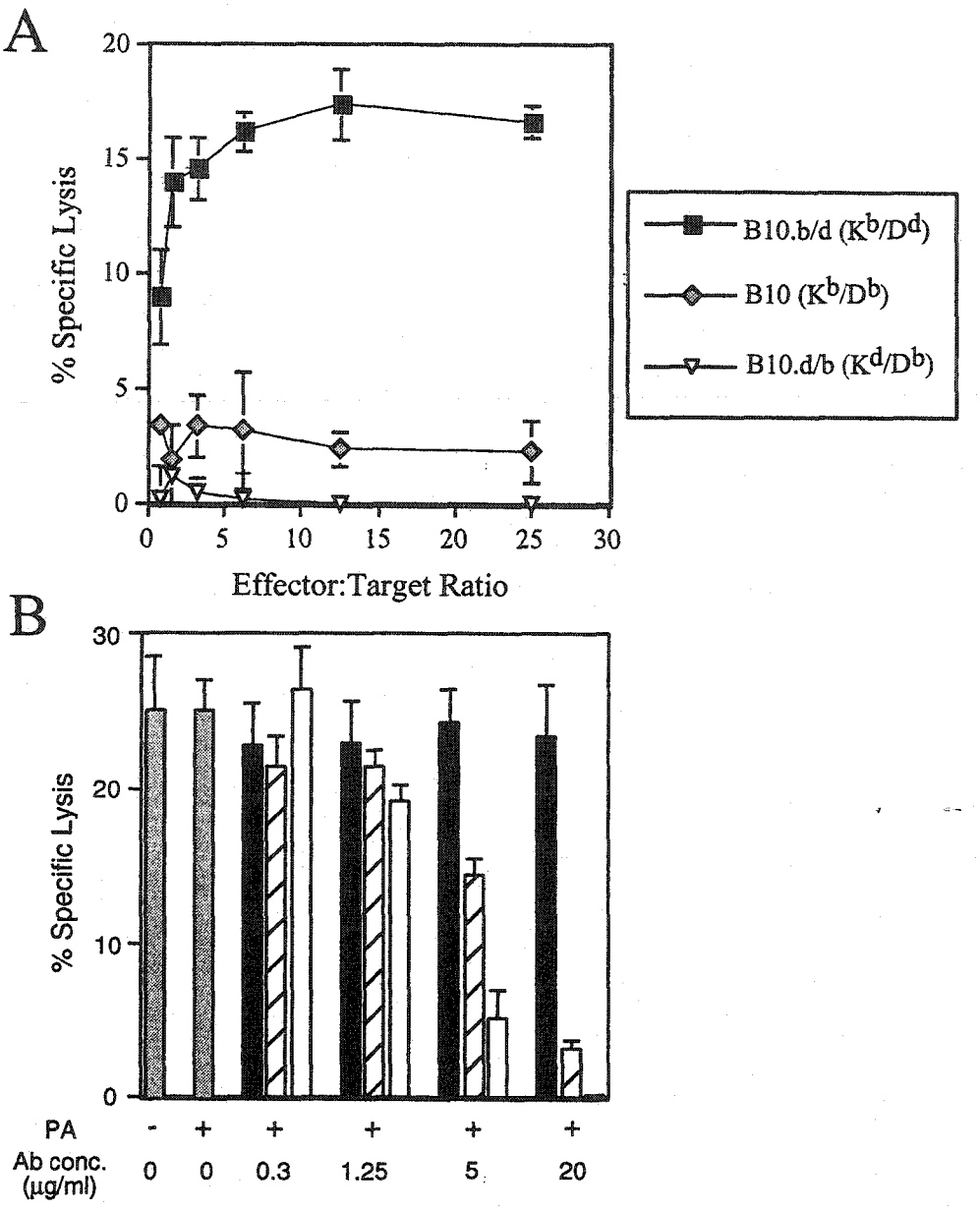

Figure 3-7. Lysis of H-2^d Con A blast targets by Ly-49W-transfected RNK cells is dependent on H-2D^d recognition. *A*, Lysis by Ly-49W-transfected RNK-16 clone 7E8 of Con A blasts generated from intra-MHC recombinants B10.d/b (H-2K^d and D^b), B10.b/d (H-2K^b and D^d) and the B10 (H-2K^b and D^b) control strain. *B*, Lysis of B10.D2 Con A blasts by 7E8 in the presence of medium or PA in control wells (gray bars), or PA plus 34-5-8S (anti-Dd α1/α2 domain, open bar), 34-2-12S (anti-D^d α3 domain, hatched bar) or isotype control antibody B27 M1 (anti-HLA B27, black bar) at the indicated concentrations. Abs were preincubated with PA (2mg/10mg of Ab) to prevent ADCC. Abs and PA remained in the wells throughout the assay. The effector:target ratio in *B* was 12.5:1. Cytotoxicity was measured after 4 h. Data are the means of triplicate wells ± SD.

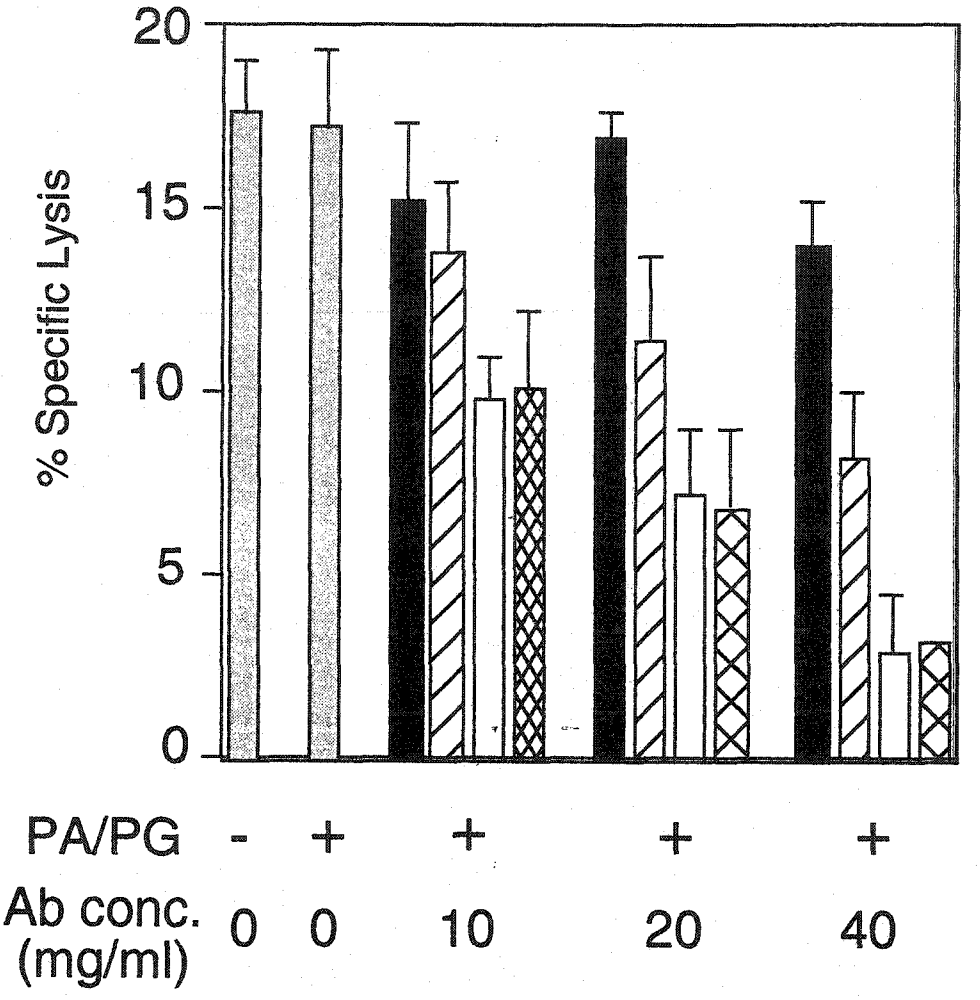

Figure 3-8. Lysis of H-2Dk-expressing targets by Ly-49W-transfected RNK-16 is inhibited by antibodies that recognize Ly-49W. Cytotoxicity of B10.BR (H-2^k) Con A T cell blasts by the Ly-49W transfected RNK-16 clone 10G5 was measured in the presence of medium or PA/PG alone (gray bars), or PA/PG with OX-8 (black bars), 4D11 (single hatched bars), Cwy-3 (open bar), or 4D11 plus Cwy-3 (double hatched bars), at the indicated concentrations. Abs were preincubated with PA/PG (4μg/10μg Ab) for 30 min prior to addition to effector cells. Effector cells were incubated with the mAbs and PA/PG for 15 min prior to the cytotoxicity assay. The PA/PG and Abs remained in the wells throughout the assay. The effector:target ratio was 12.5:1. Cytotoxicity was measured after 4 h. Date are the means of triplicate wells ± SD.

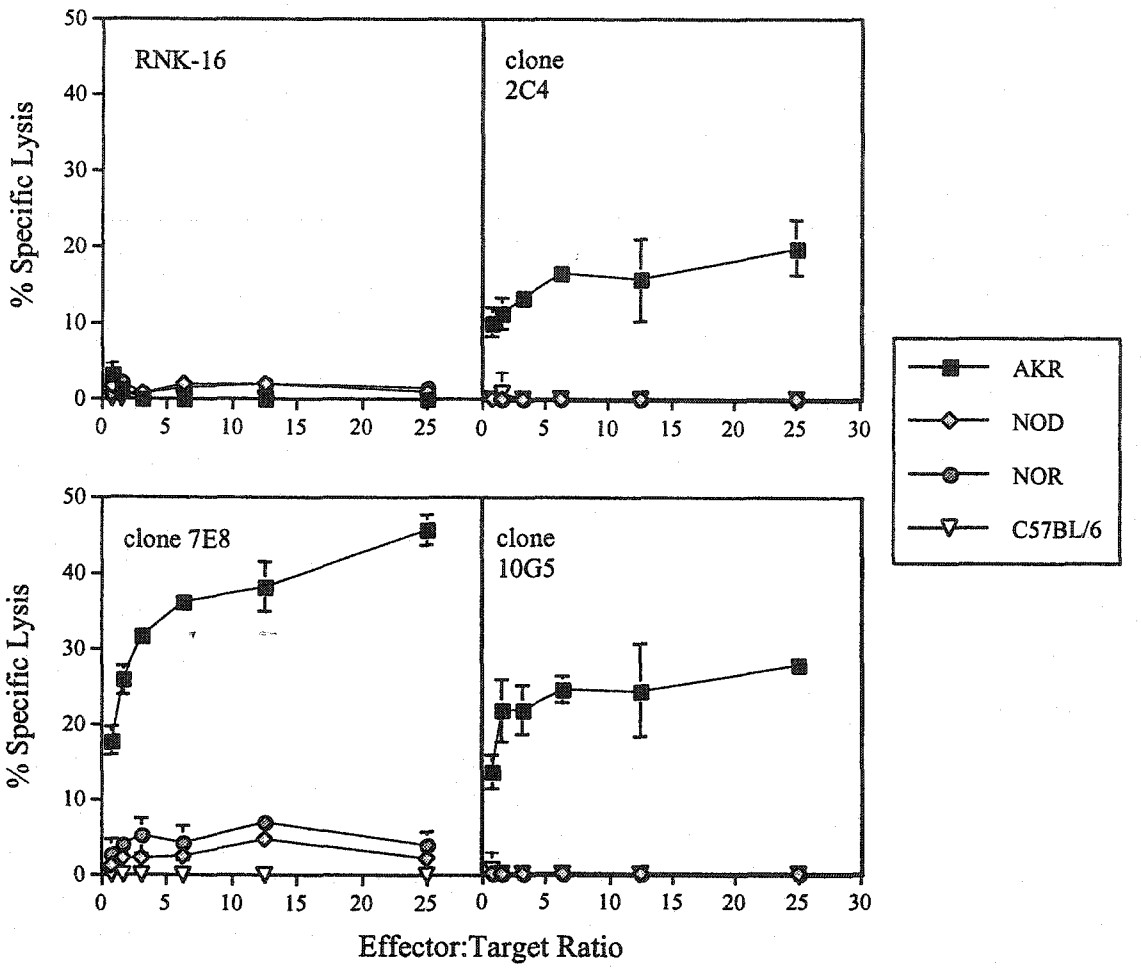

Figure 3-9. Ly-49W^{NOD} RNK-16 transfectants do not lyse NOD or NOR Con A T cell blasts. RNK-16 and individual Ly-49W^{NOD} transfectant clones 2C4, 7E8 and 10G5 were assayed for cytotoxicity against Con A T cell blasts from AKR/J, C57BL/6, NOD and NOR mouse strains, at the indicated effector to target cell ratios. Cytotoxicity was measured after 4 hr. Data are the means of triplicate wells ± SD.

CHAPTER IV

ALLELIC VARIATION IN THE ECTODOMAIN OF AN INHIBITORY LY-49 RECEPTOR ALTERS ITS SPECIFICITY FOR ALLOGENEIC AND XENOGENEIC LIGANDS [1,2]

Elizabeth T. Silver, Kerry J. Lavender, Dong-Er Gong, Bart Hazes and Kevin P. Kane

A. Introduction

Natural killer cells are mediators of innate immune responses against transformed or virally infected cells (1, 2). NK cells can also recognize allogeneic cells which may be important in bone marrow rejection (3, 4). Natural killer cells express a variety of inhibitory receptors specific for classical (class Ia) and nonclassical (class Ib) MHC-encoded molecules (1).

Regulation of NK activities can be explained in part by the "missing self" hypothesis (5). For example, NK inhibitory receptors engaged by levels of class I MHC

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proteins found on healthy cells prevent NK activation, whereas pathologically altered cells deficient in class I expression are not protected by this mechanism. In the mouse, inhibitory receptors specific for class Ia MHC molecules are encoded by the Ly-49 multigene family, which consists of many closely related genes located in the NK gene complex (NKC) on chromosome 6 (6, 7). Ly-49 receptors are type II transmembrane proteins that form disulfide-bonded homodimers (8). Inhibitory Ly-49 molecules contain an immunoreceptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic tail that becomes phosphorylated on tyrosine upon receptor engagement (9). This event recruits and activates the tyrosine phosphatase SHP-1, which disrupts membrane proximal signaling relevant to NK activation (9). Other Ly-49 family members lack the ITIM and instead contain a charged residue in the transmembrane segment for association with the immunoreceptor tyrosine-based activating motif (ITAM)-containing signaling adapter protein DAP12 (10, 11). Engagement of DAP12-associated Ly-49 receptors leads to tyrosine kinase (Syk or Zap-70) dependent NK cell activation (11).

Ly-49 receptors are allele specific in their recognition of class I ligands and are functionally, although not structurally, equivalent to killer Ig-like receptors (KIR) of human NK cells (1). The inhibitory Ly-49A and Ly-49G receptors prevent NK cytolytic activity upon recognizing H-2D^{d} (7, 12). Activating Ly-49 receptors D, P and W are also class I allele specific in their recognition (13-15), whereas the specificity of Ly-49H, another activating receptor, is unknown. Ly-49D recognizes xenogeneic ligands of rat and hamster (16, 17), while Ly-49H has a role in resistance to mouse CMV (18-20). Many Ly-49 genes including Ly-49A and Ly-49G exhibit allelic variation between mouse strains (14, 15, 21, 22). Most residues differing between Ly-49 alleles are in the

extracellular stalk and carbohydrate recognition domains (CRD), possibly affecting ligand specificity.

Here, we demonstrate that ectodomains of Ly-49G C57BL/6 (B6) and BALB/c alleles differ in their ligand specificities. The B6 and BALB/c alleles of Ly-49G both recognize H-2D^d, but only the BALB/c allele recognizes a different class I molecule, H-2D^k. Furthermore, the ectodomain of the Ly-49G$^{BALB/c}$ allele exhibits a distinct and only partially overlapping pattern of xenogeneic ligand recognition with the Ly-49G^{B6} allele. Thus, our results indicate that allelic variation in Ly-49 ectodomains can alter receptor specificity for mouse class I MHC molecules and xenogeneic ligands.

B. Materials and Methods

Animals

Five to 8 wk-old female C57BL/6, BALB/c, DBA/2, CBA/J, C57BL/10 (B10), B10.D2-H2^d/nSn (B10.D2), and B10.BR mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Six-wk-old DA, AO, F344, LEW, LOU, and PVG rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and Harlan U.K. (Bicester, U.K.). Experiments met the approval of the Animal Welfare and Policy Committee of the University of Alberta.

Hybridomas and antibodies

The following antibodies were produced from hybridomas obtained from American Type Culture Collection (Manassas, VA), except Cwy-3, which was generated

in this laboratory: 4D11 (rat IgG2a), anti-Ly-49G (12); Cwy-3 (IgG1), anti-Ly-49G (23); M1/42 (rat IgG2a), anti-mouse class I MHC (24) and Y13-238, anti p21 ras (25). Antibodies were prepared from NH_4SO_4 precipitates as described (14). Purified OX-8 (IgG1) anti-rat CD8α was purchased from BD PharMingen (San Diego, CA). FITC-coupled mouse anti-rat IgG was purchased from Jackson ImmunoResearch (West Grove, PA).

Cell lines

RNK-16, a spontaneous F344 rat strain NK cell leukemia cell line (9), was provided by Dr. M. Nakamura at the University of California at San Francisco. The RNK-16 cells were maintained in RPMI 1640 supplemented with 10% FCS, L-glutamine, penicillin, streptomycin and 5 x 10^{-5} M 2-ME. YB2/0, a non-secreting rat myeloma, was obtained from American Type Culture Collection. The Chinese hamster ovary cell line CHO was a gift from Dr. G. Armstrong at the University of Alberta, Edmonton. The YB2/0 and CHO cell lines were maintained in RPMI 1640 supplemented with 10% FCS, L-glutamine, penicillin, streptomycin, and 1 mM sodium pyruvate.

Generation of RNK-16 effector cells expressing wild type Ly-49G alleles and chimeric Ly-49WG receptors

The cDNAs encoding B6 and BALB/c Ly-49G2 alleles were prepared by RT-PCR from total RNA obtained from IL-2 activated NK cells, as described for cloning the NOD allele of Ly-49G (15). The cDNAs containing the coding region for the wild type *Ly-49G2* alleles was inserted into the *Xba*I-*Xho*I sites of the mammalian expression

vector BSRαEN (provided by Dr. Andrey Shaw, Washington University, St. Louis). To create the chimeric receptors, a *Bst*EII restriction site was silently introduced by site-specific mutagenesis at the boundary between the transmembrane and extracellular domains of both *Ly-49W2* and *Ly-49G2* cDNAs. The *XhoI*/*Bst*EII fragment of *Ly-49W2* and the *Bst*EII/*Xba*I fragment of *Ly-49G2* were simultaneously inserted into BSRαEN at the *Xho*I-*Xba*I sites to create BSR.Ly-49WG^{B6} and BSR.Ly-49WG$^{BALB/c}$. RNK-16 cells were stably transfected with individual constructs as previously described (15).

Generation of YB2/0 target cells expressing mouse class I MHC

A cDNA encoding H-2D^k was cloned by RT-PCR from the RDM4 cell line. The D^k cDNA we cloned encodes a mature D^k protein identical to that previously reported (26). The coding sequence for H-2D^k or H-2D^d was inserted into the expression vector pCI-neo (Promega, Madison, WI) and transfected into YB2/0 as described (14).

Flow cytometric analysis

Expression of Ly-49WG chimeric or full length Ly-49G allelic receptors on RNK-16 transfectant clones was measured by FACS analysis with the 4D11 mAb after blocking FcR with normal mouse serum. The secondary Ab was FITC-labeled mouse anti-rat IgG. Samples were analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Generation of Con A T cell blasts

Con A-activated T cell blasts were prepared from spleens of various mouse and rat strains. Fifteen million spleen cells were cultured at $5x10^6$ cells/ml in RPMI 1640 with 10% heat-inactivated FCS, 2-ME, and 3 μg/ml Con A (Sigma-Aldrich, St. Louis, MO) for 48 h. Blast cells were recovered after washing in RPMI 1640 medium.

Cytotoxicity assays

Target cells were labeled at 37°C with 100-150 μCi of $Na^{51}CrO_4$ (^{51}Cr) (Mandel/NEN Life Science Products, Guelph, Canada); YB2/0, YB2/0 transfectants and CHO cells were labeled for 1 h. The Con A blasts were labeled for 1.5 h. Following extensive washes, $1x10^4$ ^{51}Cr-labeled cells were incubated with RNK.WG or RNK.49G effector cells for 4 h at 37°C in V-bottom microtiter plates at various E:T ratios in triplicate. After incubation, supernatant samples were counted in a MicroBeta TriLux Liquid Scintillation Counter (PerkinElmer, Wellesley, MA). Percent specific lysis was determined as [experimental release – spontaneous release]/[maximum release – spontaneous release] x 100. In Ab inhibition experiments, mAbs were incubated for 30 min with 1 μg of soluble protein A and G (PA/PG)(Calbiochem, La Jolla, CA) for each μg of mAb, then added to effector cells for 15 min before the cytotoxicity assay to prevent reverse ADCC as described (15). The mAbs and PA/PG were present throughout the cytotoxicity assays. Each cytotoxicity experiment was performed a minimum of three separate times.

C. Results

Construction and expression of Ly-49WG chimeric receptors

The ligand specificity of Ly-49G^{B6} has been examined by Ab blocking in NK cell assays using the 4D11 Ab, and by protein binding with class I MHC tetramers (12, 27). Results from these studies suggest that Ly-49G^{B6} recognizes H-2D^{d}, although perhaps less strongly than Ly-49A does (27). Complementary DNA sequences cloned from CB.17, BALB/c, NOD/NOR and 129/J strains have nucleotide identities of 98% or greater when compared with *Ly-49G* from the B6 strain and have been designated alleles of *Ly-49G* (12, 15, 28). In addition, *Ly-49G*$^{BALB/c}$ is less than 87% identical to other *Ly-49* family members cloned from the B6 mouse, giving additional support to the BALB/c sequence being an allelic form of *Ly-49G* and not another member of the *Ly-49* family. We cloned cDNAs encoding the B6 and BALB/c *Ly-49G2* alleles by RT-PCR from IL-2 activated B6 and BALB/c NK cells and they are identical to those cloned earlier from B6 and CB.17 mice, respectively (12, 29). The extracellular domains of Ly-49G^{B6} and Ly-49G$^{BALB/c}$ differ at 9 amino acids positions; four in the membrane proximal stalk region and five in the membrane distal CRD (Fig. 4-1A). Only two other amino acids differ between B6 and BALB/c alleles, one in the transmembrane segment and one in the cytoplasmic tail (Fig. 4-1A).

Amino acid differences in the extracellular domains of Ly-49G^{B6} and Ly-49G$^{BALB/c}$ prompted us to determine whether their ligand specificities differ. Complications are associated with attempting to define specificities of individual Ly-49 receptors using NK cells. NK cells are highly heterogeneous regarding the number of

distinct Ly-49 receptors expressed, and as we and others have shown, most (if not all) antibodies originally thought to be specific for individual Ly-49 proteins crossreact with other Ly-49 molecules (14, 15, 28). Such complications can be avoided by expressing individual mouse Ly-49s on the heterologous rat NK cell line, RNK-16 (9). To examine the specific contribution of Ly-49G allele ectodomains to receptor specificity, we utilized chimeric receptors. External domains of B6 and BALB/c alleles of *Ly-49G* were fused to a common cytoplasmic/transmembrane portion of the activating Ly-49W receptor (Fig. 4-1B), creating activating receptors with the specificity of inhibitory Ly-49G proteins. An advantage of using chimeric receptors is that the functional specificity of an inhibitory receptor ectodomain can be determined directly in a positive NK response, as opposed to previous approaches reliant on Ab reversal of receptor dependent inhibition of undefined activating receptor function. Chimeric Ly-49WG constructs were stably transfected into RNK-16 cells. Three clones each of the RNK.WG^{B6} and RNK.WG$^{BALB/c}$ transfectants with matching expression levels, as determined by the B6 and BALB/c Ly-49G crossreactive Ab, 4D11, were selected for study (Fig. 4-1C). Additionally, an RNK.WG^{B6} clone expressing approximately three-fold higher levels of Ly-49WG^{B6} (Fig. 4-1C) was used in some comparisons with the RNK.WG$^{BALB/c}$ transfectants.

Ectodomains of B6 and BALB/c Ly-49G allelic forms differ in their specificities for class I MHC ligands

Lysis of Con A-activated T cell blasts from DBA/2, CBA/J and C57BL/6 mice by the RNK.WG transfectants was determined. RNK.WG^{B6} recognizes DBA/2(H-2^{d}) Con A blasts (Fig. 4-2A), consistent with previous studies indicating that Ly-49G^{B6} recognizes

H-2D^{d} (12, 30). RNK.WG$^{BALB/c}$ also recognizes DBA/2 Con A blasts (Fig. 4-2A). In addition, we consistently observe stronger recognition with RNK.WG$^{BALB/c}$ over RNK.WG^{B6} with multiple clones having matched levels of Ly-49WG expression, e.g. clone 4G8^{B6} and 1G9$^{BALB/c}$ (Fig. 4-2A, and data not shown). These results may indicate that Ly-49G$^{BALB/c}$ has a higher affinity than Ly-49G^{B6} for H-2^{d} ligand(s) and/or other DBA/2 ligands. We also find that RNK.WG$^{BALB/c}$ displays equally effective recognition of CBA/J and DBA/2 Con A blasts (Fig. 4-2A). This is in striking contrast to RNK.WG^{B6}, which even with increased Ly-49WG expression, e.g. clone 1E12, shows very little or no recognition of CBA blasts (Fig. 4-2A). These results suggest that Ly-49G$^{BALB/c}$ recognizes one or more CBA expressed ligands, possibly H-2^{k}-encoded, while Ly-49G^{B6} does not. Neither chimeric receptor mediated lysis of B6 Con A blasts (Fig. 4-2A), which is consistent with previous studies of Ly-49G^{B6} specificity (12, 27). Untransfected RNK-16 did not lyse any of the Con A blasts (data not shown, and (15)).

To determine whether H-2^{k} MHC molecule(s) are recognized by Ly-49WG$^{BALB/c}$ and not by the WG^{B6} chimera, we examined lysis of B10 congenic T cell blasts, differing only in their MHC haplotype, by B6 and BALB/c Ly-49WG chimeric receptor transfectants matched for receptor expression. Since substantial and comparable cytotoxicity was observed with B10.D2 and B10.BR, but not B10 targets, we conclude that the BALB/c ectodomain recognizes allogeneic H-2^{k} as well as syngeneic H-2^{d} ligand(s) (Fig. 4-2B). In contrast, only the H-2^{d}-expressing B10.D2 target was recognized by the Ly-49WG^{B6} chimera (Fig. 4-2B). To identify specific MHC class I ligands that may be recognized by the Ly-49G ectodomains, we expressed H-2D^{d} and H-2D^{k} in rat YB2/0 cells. While RNK-16 cells can lyse YB2/0 at moderate levels,

augmented killing of YB2/0 mouse class I MHC transfectants indicates class I specificity of mouse activating Ly-49 receptor transfectants of RNK-16 cells (13, 15). Our results confirm that Ly-49WG^{B6} recognizes H-2D^{d} but not H-2D^{k}, while Ly-49WG$^{BALB/c}$ recognizes H-2D^{d} and H-2D^{k} (Fig. 4-2C). Enhanced lysis is not observed against mouse class I-transfected YB2/0, compared to untransfected YB2/0 using untransfected RNK-16 (data not shown). We verified that specificity for T cell blast ligands is conferred by the chimeric mouse Ly-49 receptors expressed by the RNK effectors. Antibodies recognizing the B6 (Cwy-3) or BALB/c (4D11) Ly-49G ectodomains completely or substantially blocked recognition of DBA/2(H-2^{d}) or CBA(H-2^{k}) targets by the Ly-49WG^{B6} and Ly-49WG$^{BALB/c}$-expressing RNK-16 effector cells, respectively (Fig. 4-3). We conclude that the ectodomains of two allelic forms of Ly-49G differ in their specificities for mouse class I MHC ligands.

We went on to determine whether wild type inhibitory B6 and BALB/c Ly-49G allele products could corroborate our observations with the chimeric Ly-49 receptors. To this end, we expressed each Ly-49G allele product in RNK-16 cells and examined cytotoxicity by these effector cells against YB2/0 cells or YB2/0 cells transfected with H-2D^{d} or D^{k} (Fig. 4-4). Three clones expressing each Ly-49G allele were compared. Very modest inhibition of cytolysis of the H-2D^{d}-expressing target was observed with one RNK.G^{B6} clone, 1B6, and the other two such clones, 5D10 and 6A1, were not inhibited by D^{d} (Fig. 4-4A). All three RNK.G^{B6} clones were not inhibited by target cell H-2D^{k} expression (Fig. 4-4A). In contrast, inhibition of cytolysis was readily detected with targets expressing D^{d} or D^{k}, using all three RNK.G$^{BALB/c}$ effector cells (Fig. 4-4B). All target cells were lysed equally well by untransfected RNK-16 cells (data not shown). The

wild type Ly-49G$^{BALB/c}$ allele product clearly demonstrated the same class I MHC specificity as the chimeric Ly-49GWG$^{BALB/c}$ receptor and confirmed the specificity of this Ly-49G allele. The relative insensitivity of the wild type Ly-49G^{B6} allele to expression of the H-2D^{d} product is consistent with a previous report that this allele has only very weak interactions with H-2^{d} expressing cells or H-2D^{d} tetramers (27). Therefore, its class I specificity in the form of an inhibitory receptor may not be readily detectable using the RNK system. However, the class I MHC specificity of the chimeric Ly-49WG^{B6} activating receptor does indicate D^{d} recognition and this is in agreement with the specificity suspected for Ly-49G expressed on NK cells (12). The difference in the extent of D^{d} recognition by the wild type Ly-49G^{B6} as opposed to its chimeric receptor may be that a somewhat greater sensitivity is obtainable with the activating form of the receptor. No evidence is found for D^{k} recognition by the Ly-49G^{B6} allele in either form of the receptor.

B6 and BALB/c Ly-49G ectodomains differ in their specificities for xenogeneic ligands

In addition to recognizing D^{d}, the activating mouse Ly-49D receptor can stimulate NK mediated lysis by recognizing undefined xenogeneic ligands expressed on certain hamster and rat targets (16). For example, Ly-49D does not recognize a ligand from Syrian hamsters but does recognize a ligand from Chinese hamsters, as evidenced by lysis of CHO cells (16). Our results show that CHO cells are also recognized by the BALB/c allele of Ly-49G, with all three clones expressing the Ly-49WG$^{BALB/c}$ chimeric receptor demonstrating substantial CHO cytotoxicity (Fig. 4-5A). In contrast, three clones expressing equivalent levels of the Ly-49WG^{B6} chimeric receptor showed no lysis

of CHO targets (Fig. 4-5A). Even when the Ly-49WG^{B6} chimeric receptor is expressed at higher levels, such as with clone 1E12, CHO cells are not recognized despite significant lysis of DBA/2 blasts in the same assays (Fig. 4-5B). These results prompted examination of Ly-49G allele recognition of rat xenogeneic ligands. Con A blasts of the PVG strain were lysed by RNK.WG^{B6} and RNK.WG$^{BALB/c}$ effector cells, indicating that ectodomains of either Ly-49G allele are able to recognize a rat ligand (Fig. 4-6A). RNK cells expressing the Ly-49WG$^{BALB/c}$ chimera lysed LEW strain Con A blasts, whereas those expressing the Ly-49WG^{B6} chimera were unable to do so (Fig. 4-6A), suggesting another difference in the specificities of the two Ly-49G alleles. T cell blasts from several other rat strains, including AO, LOU, DA, and F344, were not recognized by either Ly-49G allele, despite significant recognition of DBA/2 Con A blasts in the same assays (Fig. 4-6B). No lysis of CHO or rat blasts was observed with RNK-16 cells (data not shown). Thus, B6 and BALB/c ectodomains of Ly-49G differ not only in recognition of mouse class I MHC, but also xenogeneic hamster and rat ligands.

D. Discussion

A number of Ly-49 receptors including Ly-49A, C, and G demonstrate allelic variation (14, 15, 21, 22, 31). When allelic differences occur in extracellular ligand-binding regions, there is the possibility that the affinity, specificity, or both, of Ly-49 ligand binding could be affected. The first study attempting to address this question compared B6 and BALB/c alleles of Ly-49C and found no difference in class I binding specificity (27). These two alleles have only one variance in the CRD, a rather conservative difference of Phe vs Tyr, and thus would very likely have identical ligand

specificities. Another report by Mehta et al. provided evidence for variability in the affinity of different Ly-49A alleles for H-2D^d through quantitative differences in D^d tetramer binding to Ly-49A alleles expressed by transfection on CHO cells (21). They observed partial or no inhibition of NK lysis mediated by D^d with Ly-49A$^+$ NK cells, defined by Ab staining, from BALB/c and SWR strain mice, respectively. In contrast, complete inhibition was observed with such NK cells from two other mouse strains. Such results suggest a hierarchy of Ly-49A allelic interaction with D^d and are consistent with our findings where the BALB/c allele of Ly-49G appears to recognize D^d somewhat more efficiently than the B6 allele. More importantly, however, we found that the ectodomain of the BALB/c Ly-49G allele confers the gain of specificity for another class I ligand, H-2D^k, as well as conferring recognition of a distinct and only partially overlapping repertoire of xenogeneic ligands compared with the B6 allele. A potential limitation of studies using NK subsets defined by Ab staining to determine the specificity of individual Ly-49 receptors is the substantial complication of Ab crossreactivity with other Ly-49 receptors, defined or undefined, that may be expressed on the NK cells being examined. Differences in ligand specificity between the BALB/c and B6 alleles of Ly-49G, identified here, can be directly attributed to differences in their ectodomains, since our tests included transfection of individual chimeric Ly-49 constructs which limited receptor variation to the Ly-49G extracellular domains.

Since our studies principally used chimeric receptors, there is the potential that specificities of intact Ly-49G alleles could be different from those we have identified, due to polymorphism in the transmembrane segment/cytoplasmic tail, or in using an activating as opposed to inhibiting form of the receptor. When we expressed the wild

type BALB/c allele of Ly-49G in RNK-16, it was found to have the same pattern of MHC recognition as the $WG^{BALB/c}$ chimeric receptor, with inhibition of RNK-16 mediated cytotoxicity against targets expressing H-$2D^d$ and H-$2D^k$ being observed. In contrast, RNK-16 transfected with the B6 allele of Ly-49G exhibited relatively unaltered killing of H-$2D^d$ or D^k expressing target cells. As we have indicated, the B6 Ly-49G allele was previously reported to have only weak interactions with H-2^d targets or H-$2D^d$ tetramers and no interactions with H-2^k expressing cells or H-$2D^k$ tetramers (27). It is possible that its specificity in the form of an inhibitory receptor may not be readily detectable using the RNK system. However, the specificity of the chimeric Ly-$49WG^{B6}$ activating receptor does agree with wild type Ly-$49G^{B6}$ expressing mouse NK cells and Ly-$49G^{B6}$ transfectants (12, 27), perhaps due to somewhat greater sensitivity obtained using the chimeric activating receptor. The influence of the two amino acid residues, one in the transmembrane segment, and one in the cytoplasmic tail, that differ between B6 and BALB/c Ly-49G alleles, on receptor specificity or function were not investigated here. We consider it unlikely that they will have a significant impact on receptor specificity or function. The former is a conservative change and the latter, although a nonconservative change, occurs at a substantial distance from the ITIM sequence (Fig. 4-1A).

The co-crystal of Ly-49A bound with D^d may serve as a model for Ly-49-class I MHC interactions (32). Two independent sites of Ly-49A interaction on D^d were identified: site 1 at the α1 and α2 domain junction, and site 2 in the cleft formed by the α1/α2 domains, the conserved α3 domain and beta-2 microglobulin (Fig. 4-7). Of CRD residues differing between Ly-$49G^{B6}$ and Ly-$49G^{BALB/c}$, only residue 246 interacts

directly with class I and it does so at both sites 1 and 2 (Fig. 4-7). Ly-49A and Ly-49G^{B6} have aspartate at position 246 which forms salt bridges with class I residues Arg170 (site 1) and Lys243 (site 2) (Fig. 4-7). Ly-49G$^{BALB/c}$ cannot make either salt bridge due to the Asp246→Gly substitution. Molecular modeling suggests that this loss cannot be compensated for in site 2. Consequently, if Ly-49 Asp246 interaction with Lys243 at site 2 is important for receptor engagement, Ly-49G$^{BALB/c}$ is predicted to recognize D^d and D^k more poorly, but this is not observed (Fig. 4-2). At the site 1 interface, the Asp246→Gly substitution can be compensated for by side chain rearrangement of Asp244 as we proposed previously for interaction with D^k by the Ly-49G-related Ly-49W activating receptor (33). The Asp246→Gly substitution creates space and enhances backbone flexibility. Both effects may increase the ability to adapt to potential ligands, thereby giving rise to a broader class I specificity as is observed (Fig. 4-2). However, additional studies are required to determine the relative physiological importance of site 1 and site 2 in Ly-49G interactions with class I MHC ligands.

We provide evidence that the BALB/c allele of Ly-49G recognizes the xenogeneic hamster target CHO. Ly-49G$^{BALB/c}$ and the activator Ly-49D may share undefined CHO ligand(s), or recognize different CHO ligands. It was reported that 4D11$^+$ NK cells of B6 origin do not lyse CHO cells unless incubated with the 4D11 Ab, suggesting that Ly-49G^{B6} may recognize CHO ligands (34). In contrast, using the Ly-49WG^{B6} allele chimeric receptors expressed on RNK cells, we do not observe recognition of CHO cells, suggesting that the ectodomain of Ly-49G^{B6} is not capable of recognizing CHO ligands. A complication for the 4D11$^+$ NK experiments described previously is that Ly-49A, and possibly other Ly-49 receptors also are recognized by the

4D11 Ab (35, 36), potentially obscuring which NK receptor(s) are recognizing CHO ligands.

Ly-49D recognizes xenogeneic rat Con A blast targets from the F344 and LEW strains, but not from PVG (16). In contrast, we found that both B6 and BALB/c Ly-49G ectodomains recognize PVG, but not F344. These results suggest that Ly-49G and Ly-49D can recognize different rat ligands. Rat MHC molecules may be ligands for mouse Ly-49D (16), and it is possible that this is also the case for Ly-49G. The ectodomain of Ly-49G$^{BALB/c}$ recognizes LEW targets having the rat RT1^{l} MHC haplotype, but not F344 targets that have the RT1^{lv1} MHC (Fig. 4-6A, B). LEW and and F344 rats are genetically identical in RT1.A and RT1.B/D (classical class I and class II MHC) genes, but differ in RT1.C/E/M regions of the rat MHC (37, 38). If Ly-49G$^{BALB/c}$ recognizes a rat MHC ligand, then a non-classical molecule encoded in the RT1.C/E/M region of the RT1^{l} haplotype may be a ligand. Rat non-classical class I molecules encoded in the RT1.C/E/M region of RT1^{l} can activate or inhibit rat NK cell function (39-41). The significance of xenoantigen recognition by Ly-49 receptors is not understood but could relate to interspecies predation and resistance to pathogen passage across species (16, 42).

Allelic exclusion, where only one or the other allele of Ly-49 genes is expressed in the majority of F1 NK cells, has been observed (43-46). The functional significance of Ly-49 allelic exclusion is unknown. However, should Ly-49 alleles have distinct ligand specificities, then Ly-49 allelic exclusion may enhance the number of distinct NK subpopulations with different ligand specificities in heterozygous animals. Our demonstration that Ly-49 alleles can show different specificity patterns for mouse class I proteins and xenogeneic ligands is consistent with this possibility. Ly-49 polymorphism

and allelic exclusion may operate together to augment NK cell functional diversity using a limited set of receptor genes.

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F. Author Contribution to Data

Elizabeth Silver cloned by RT-PCR H-2D^k, H-2L^d, the C57BL/6 and BALB/c *Ly-49G* transcripts, generated the Ly-49WG chimeric receptors, performed the COS transient transfections, generated the stable Ly-49 and class I transfectant clones, and did the flow cytometric analyses for this study. Kerry Lavender performed the cytotoxicity assays for Fig. 4-5B and Fig. 4-6, while Dong-Er Gong carried out the remainder of the cytotoxicity assays published in this study. Bart Hazes did the molecular modeling of Ly-49 described in the discussion.

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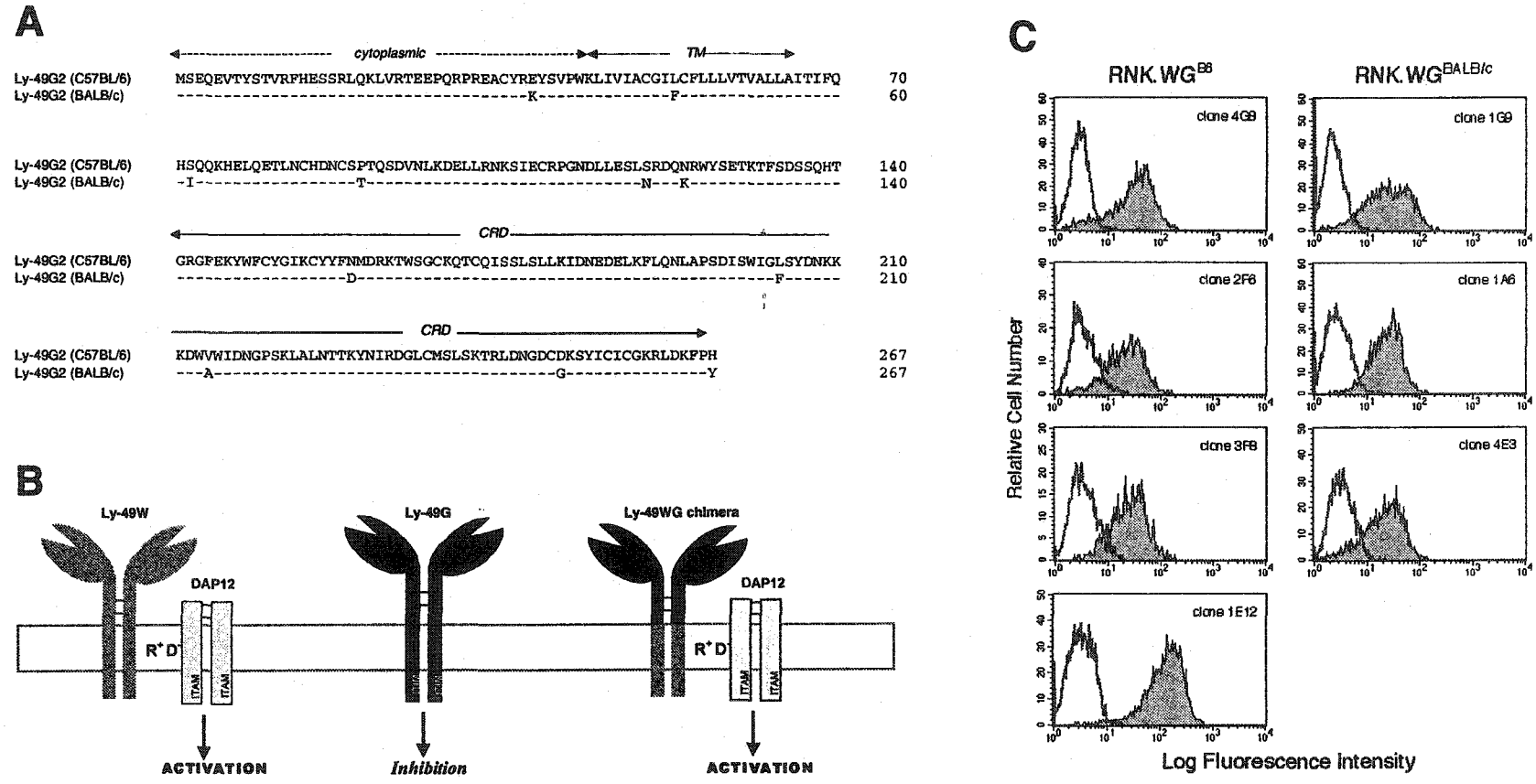

FIGURE 4-1. Construction and expression of activating Ly-49 receptors with the extracellular domain of the inhibitory receptor Ly-49G2. (*A*) Protein sequence alignment of Ly-49G B6 and BALB/c alleles. (*B*) Ly-49W cytoplasmic/transmembrane domains fused to the ectodomain of B6 or BALB/c Ly-49G create Ly-49WG chimeric receptors. (*C*) RNK-16 transfectant clones stably expressing Ly-49WG (RNK.WG) stained with the 4D11 (shaded) or isotype control M1/42 (unshaded) mAb.

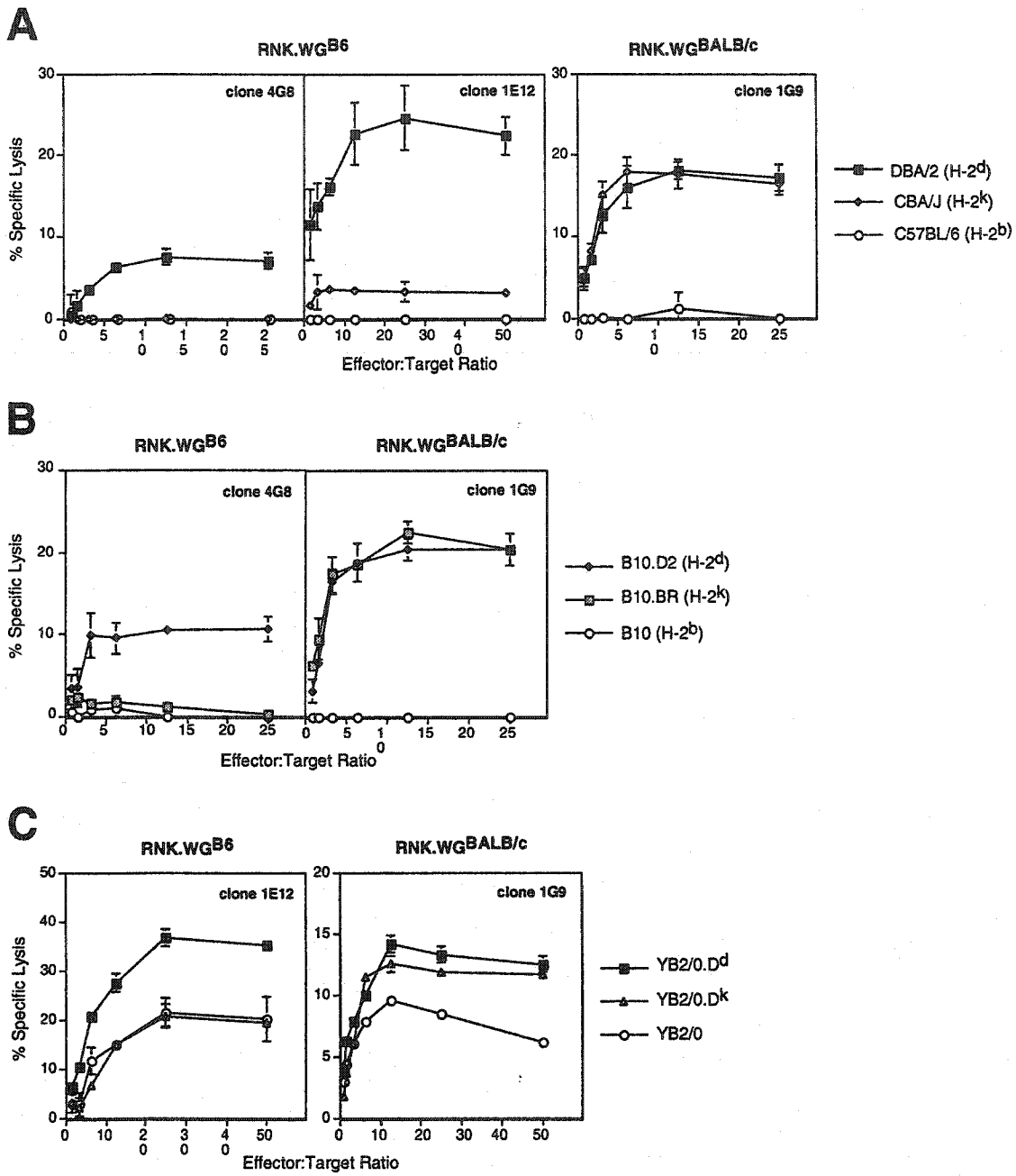

FIGURE 4-2. B6 and BALB/c Ly-49G chimeric receptors differ in their specificities for mouse class I MHC ligands. RNK.WG^{B6} and RNK.WG$^{BALB/c}$ were tested in standard 4-hour cytotoxicity assays, at the indicated E/T ratios, against DBA/2, CBA/J and C57BL/6 Con A blasts (*A*), B10, B10.D2 and B10.BR Con A blasts (*B*), or YB2/0 and YB2/0 H-2Dd or H-2Dk transfectants (*C*). Data represent the mean of triplicate wells ± SD.

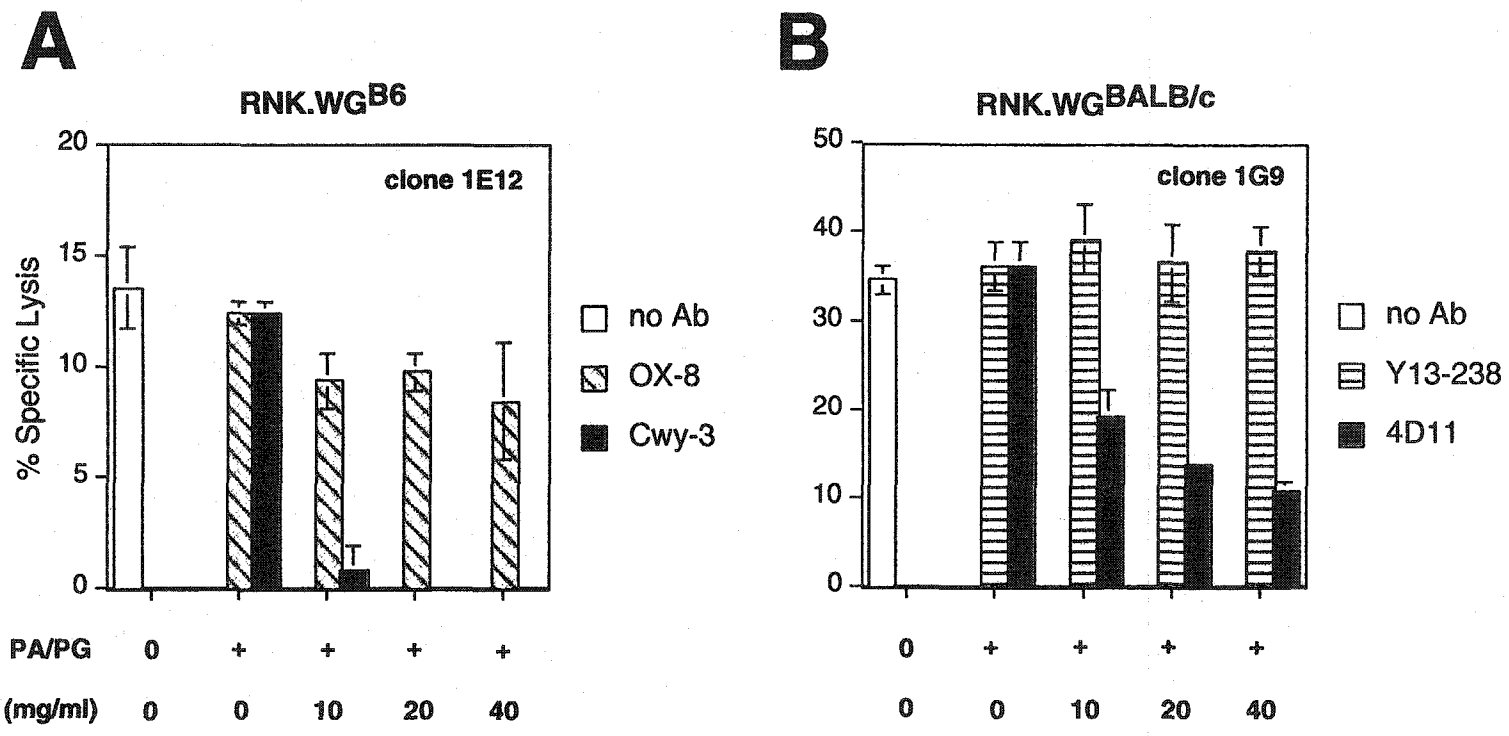

FIGURE 4-3. Lysis of DBA/2 Con A blasts mediated by the WG^{B6} Ly-49G chimeric receptor (*A*) and lysis of CBA/J blasts mediated by the $WG^{BALB/c}$ chimeric receptor (*B*) is blocked by the Cwy-3 (anti-Ly-$49G^{B6}$-specific) and 4D11 (pan Ly-49G-specific) antibodies, respectively. Lysis of DBA/2 Con A blasts by $RNK.WG^{B6}$ clone 1E12, or CBA Con A blasts by $RNK.WG^{BALB/c}$ clone 1G9, was measured with no Ab or in the presence of anti-Ly-49G Ab (Cwy-3 or 4D11, respectively) or control Abs (OX-8 or Y13-238, respectively), at the indicated concentrations. Antibodies were preincubated with 1 mg protein A/protein G (PA/PG) per mg Ab for 30 min before they were added to effector cells for 15 min. Subsequent cytotoxicity was determined at an E/T ratio of 12.5:1 in 4-h ^{51}Cr-release assays. Data represent the mean of triplicate wells $\pm$ SD.

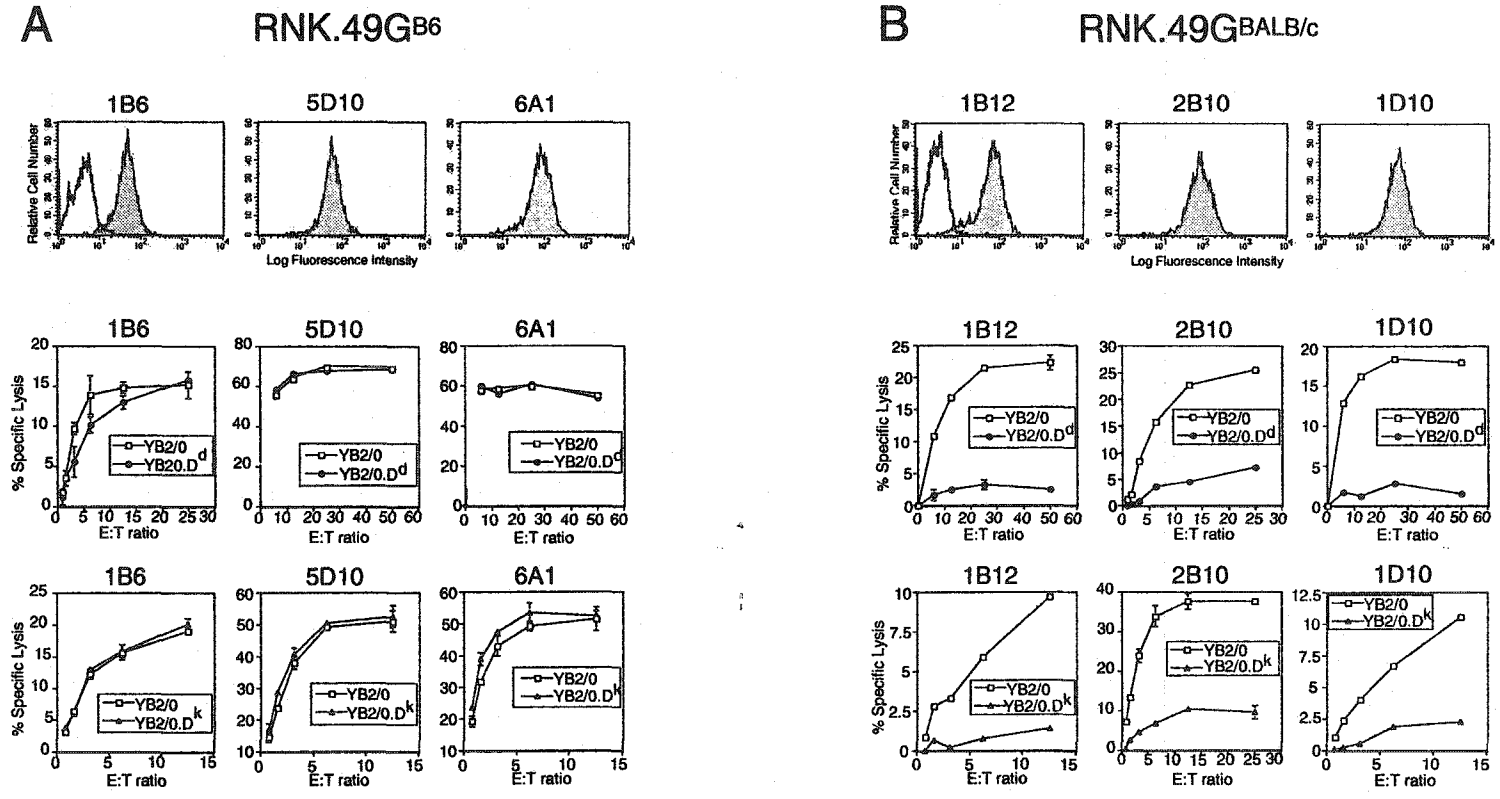

FIGURE 4-4. The BALB/c allele, but not the B6 allele of Ly-49G, strongly inhibits RNK-16 mediated lysis of H-2D^d and H-2D^k expressing target cells. RNK-16 cells transfected with the C57BL/6 allele of Ly-49G, clones 1B6, 5D10 and 6A1 (*A*), or the BALB/c allele of Ly-49G, clones 1B12, 2B10 and 1D10 (*B*), were examined for Ly-49G expression and lysis of YB2/0 cells or YB2/0 cells transfected with mouse H-2D^d or H-2D^k. RNK-16 transfectants were stained with 4D11 (shaded) and in some cases, an isotype control M1/42 to determine background staining (unshaded). Lysis was determined in standard 4-h cytotoxicity assays using the indicated E/T ratios. Data represent the mean of triplicate wells $\pm$ SD.

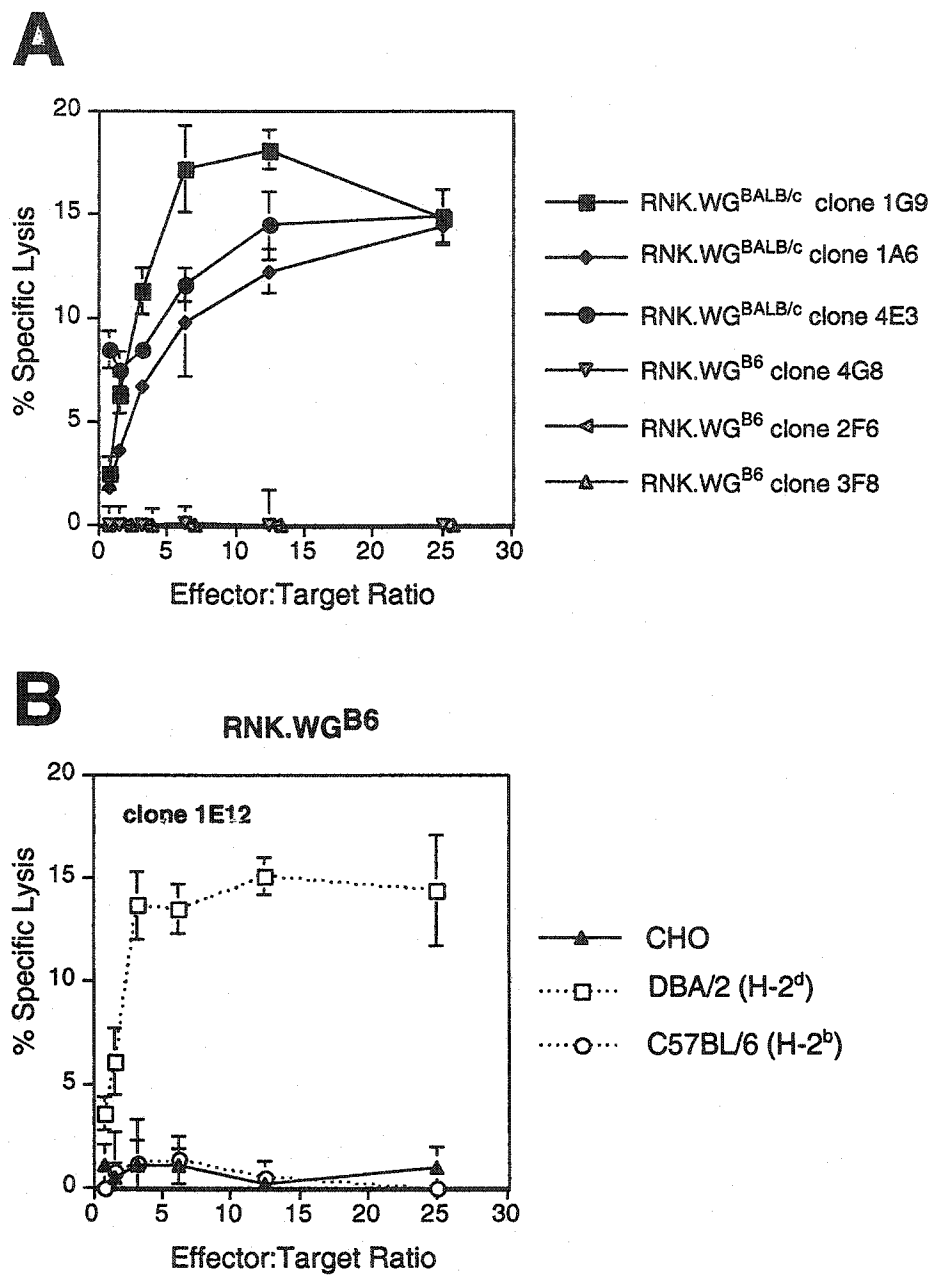

FIGURE 4-5. The ectodomain of the BALB/c but not the B6 allele of Ly-49G recognizes the xenogeneic CHO hamster target cell. Three clones each of RNK.WG^{B6} and RNK.WG$^{BALB/c}$ matched for receptor expression level were tested for cytotoxicity against CHO cells (*A*). The RNK.WG^{B6} clone 1E12 expressing high levels of the WG^{B6} receptor was also tested for cytotoxicity against CHO cells as well as DBA/2 and B6 Con A blasts (*B*). Effector cells were incubated with target cells in standard 4-hour cytotoxicity assays at the indicated E:T ratios. All data represent the mean of triplicate wells + SD. Cytotoxicity assays for (*B*) were done by K. J. Lavender.

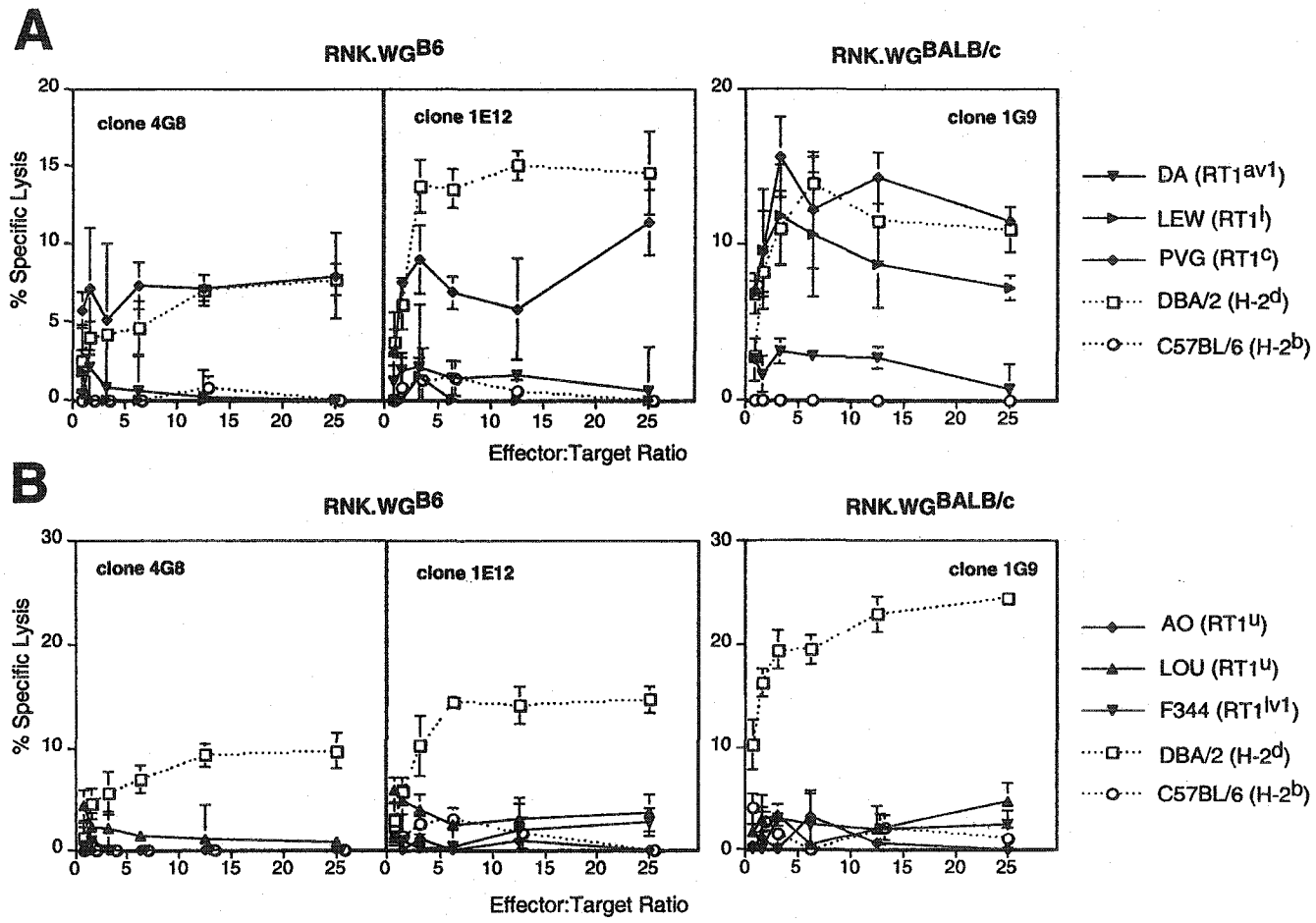

FIGURE 4-6. The B6 and BALB/c Ly-49G ectodomains exhibit only partially overlapping specificities for rat ligands. The RNK.WG^{B6} clones 4G8 and 1E12, and RNK.WG$^{BALB/c}$ clone 1G9 were used as effector cells against Con A T-cell blasts generated from splenocytes of various rat strains; DA, LEW, and PVG (*A*), or AO, LOU, and F344 (*B*), with mouse DBA/2 and C57BL/6 Con A blasts used as positive and negative controls, respectively (*A* and *B*). Standard 4-h cytotoxicity assays were performed at the indicated E/T ratios. All data represent the mean of triplicate wells ± SD. All cytotoxicity assays in this figure were done by K. J. Lavender.

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CHAPTER V

GENERAL DISCUSSION AND CONCLUSIONS

A. The Role of Ly-49 Activating Receptors

The "missing self" hypothesis proposes that NK cells recognize and kill target cells with diminished class I MHC cell surface expression. This hypothesis asserts that inhibitory receptors for class I expressed by NK cells restrain NK cytolysis only when the target cell expresses normal levels of self class I. The existence of class I receptors that recognize the same class I ligands as the inhibitory receptors, but that activate NK cytolysis upon binding to their class I ligands, would appear to contravene the "missing self" hypothesis. Speculation of the functions of activating receptors (Fig 5.1) have included that they may (a) regulate the function of co-expressed inhibitory receptors with the same class I ligand specificity by recruiting tyrosine kinases to activate the ITIM of the inhibitory receptor; (b) work with co-expressed inhibitory receptors that recognize different self class I ligands in order to identify target cells that have selectively lost one class I molecule; (c) control NK functions in coordination with inhibitory receptors, where inhibitory receptors would regulate NK cytotoxicity, while activating receptors would regulate different pathways, such as cell proliferation, and/or (d) recognize non-self or "pathogenic self" class I ligands, such as virally infected target cells expressing self class I presenting viral peptides, or virally-encoded class I homologs that interact with inhibitory receptors and down-regulate NK cytotoxicity of virally infected targets

(1). Many recent pieces of information can be integrated to try to determine the roles of activating Ly-49 receptors.

The generation of mice without functional DAP12 has helped to define those functions that rely on Ly-49 activating molecules, since the activating Ly-49 receptors must associate with the ITAM-bearing DAP12 protein for cell surface expression and signal transduction (2-6). In DAP12 deficient mice, it was demonstrated that Ly-49 activating receptors cannot substitute another signaling molecule in the absence of DAP12 (7). Mice with non-functional DAP12 have normal NK development and the expression and function of the inhibitory Ly-49 receptors appear unaffected (7, 8). This indicates that the activating Ly-49 receptors probably play no role in the regulation of inhibitory receptors by enhancing ITIM phosphorylation.

We have defined ligand specificity for two activating receptors, Ly-49P and Ly-49W from the NOD mouse strain (4, 5). While both receptors recognize mouse class I, neither recognizes self class I since transfectants of the rat NK cell line RNK-16 do not lyse NOD cells or mouse Con A blasts expressing the NOD class I antigens K^d or D^b. Likewise, the C57BL/6 allele of Ly-49D does not recognize either self class I proteins K^b or D^b, but the non-self class I D^d (9). As well, the activating receptors Ly-49D, P, and W all demonstrate specificity for H-$2D^d$ but cannot be detected by RT-PCR in wild-type BALB/c (H-2^d) NK cells (unpublished). Instead, I have found that BALB/c expresses transcripts for Ly-49L, a receptor identical to Ly-49W for the first 5 exons, but with different exons 6 and 7 (5) and this change modifies residues in a key contact site with H-$2D^d$ (10), which may diminish or destroy the ability of Ly-49L to bind D^d. Also, the sequence change removes the single N-linked glycosylation site present in Ly-49W, and

the resulting lack of glycosylation sites may eliminate Ly-49L cell surface expression. *N*-linked glycosylation has been found to be necessary for the mammalian cell surface expression of many, but not all, proteins (11-13). Ly-49L protein may not be transported to the cell surface, since Ly-49L-transfected cells express protein that can be immunoprecipitated with an antibody that recognizes Ly-49L while no cell surface expression can be detected (14) and several attempts at transfection of RNK-16 cells with *Ly-49L* has failed to generate RNK-16 cells with cell surface expression of Ly-49L (unpublished). Nonetheless, the complete range of Ly-49 receptors expressed in the BALB/c mouse has not been determined, and it has been shown that D^d mice can express Ly-49D (15, 16), but the lack of self reactivity of the activating receptors Ly-49D, P, and W suggests that activating receptors do not always recognize self class I. For this reason, activating Ly-49 receptors may not have a major function in recognizing pathogenic decreases (or increases) in the expression of all or a subset of self class I molecules, either in cooperation with inhibitory receptors or alone.

We have shown that Ly-49P recognizes the allogeneic mouse class I ligand H-$2D^d$ (4) as was previously shown for Ly-49D (9), while Ly-49W binds both D^k and D^d strongly, although D^k with much higher avidity (5). Ly-49D was shown to be responsible for the ability of C57BL/6 mice to lyse Chinese hamster ovary (CHO) cells (17, 18), and it was recently reported that Ly-49D was able to recognize a CHO class I molecule (19). The activating receptor Ly-49W also has the ability to recognize and mediate killing of CHO cells while Ly-49P does not (KJ Lavender, ET Silver, DE Gong, KP Kane, unpublished), which demonstrates that some, but not all, Ly-49 activators recognize ligands on CHO cells. Ly-49D can also kill Con A blasts from some, but not all, rat

strains (17), a capability also shared by Ly-49W which recognizes a different panel of rat strain ligands than does Ly-49D (KJ Lavender, ET Silver, DE Gong, KP Kane, unpublished). Thus, it appears that one phenomenon of Ly-49 activating receptors is to recognize non-self class I MHC, including allogeneic and xenogeneic class I. Animals could be inoculated with xenogeneic cells through predatory, aggressive or defensive behavior, and it has been suggested that the ability to eliminate xenogeneic cells might also serve to limit cross-species transmission of viruses and other pathogens (20). However, this explanation would not explain why a mouse would possess a system to manage pathogen transmission from some strains of mice, rats, and hamster, and not all strains of those species. A more feasible evolutionary option would be the development of a more direct anti-viral system. Investigation of the C57BL/6 and 129/J *Ly-49* gene clusters has found that functional genes encoding inhibitory Ly-49 receptors greatly outnumber those for activating Ly-49 receptors, but pseudogenes for activating receptors are numerous while no pseudogenes encoding inhibitory receptors have been found (5, 21-23). It has been suggested that activating Ly-49 genes may be more rapidly evolving to defend against new pathogen variants (23).

The activating receptor Ly-49H expressed in the MCMV-resistant C57BL/6 mouse mediates the recognition of cells infected with the virus MCMV (24-26), and the ligand was recently determined to be the MCMV-encoded protein m157 (27). The MCMV virus down-regulates cell surface expression of class I, and the MHC-like m157 protein acts as a "molecular decoy" to allow MCMV to evade NK-mediated clearance in MCMV-susceptible mouse strains by binding to an inhibitory receptor (27).

As yet, Ly-49H is the only activating Ly-49 receptor demonstrated to recognize viral antigens, but the relatively weak recognition of Ly-49D with mouse and rat antigens may indicate that its actual ligand has not yet been established. Resistance to the lethal mousepox virus (ectromelia) has been linked to the NKC, with C57BL/6 mice exhibiting ectromelia resistance while DBA/2 mice (and most other mice) are susceptible (28). Although a specific Ly-49 activating receptor has not been implicated in ectromelia resistance, *Nkc* allotype analysis suggests that the Ly-49 genetic regions of C57BL/6 and DBA/2 mice differ (29). It is possible that a C57BL/6 activating receptor is responsible for mediating resistance to ectromelia. It has been demonstrated that DBA/2 mice express little, if any, cell surface expression of Ly-49D (30), while there has been no analysis of DBA/2 expression of Ly-49H or any other activating Ly-49 receptors. NK cells have been shown to target hematopoietic cells preferentally over epithelial cells (31, 32). This suggests that the NCR receptors that mediate NK cell natural cytotoxicity may recognize ligands expressed only by hematopoietic cells. Ly-49D$^+$ NK cells can kill CHO targets (17, 18), which are epithelial cells. If activating Ly-49 receptors recognize viral gene products, their expression would allow NK cells to lyse virally infected cells, whether epithelial or hematopoietic in origin. Additional investigation will be required to reveal whether activating Ly-49 receptors other than Ly-49H recognize viral antigens.

In conclusion, the analysis of the current knowledge in the field suggests that activating Ly-49 receptors may in large part recognize non-self class I, such as allogeneic, xenogeneic, or virally-encoded MHC decoys. In those instances where activating Ly-49 receptors do not recognize self class I, their expression does not necessitate a reworking of the "missing self" hypothesis.

B. Ligand Binding Affinities of Activating Ly-49 Receptors

T cell activation is regulated by the CD28 and CTLA-4 receptors (33). The co-stimulatory CD28 receptor is constitutively expressed on resting T cells and augments their activation by TCR engagement of antigen on APCs. The inhibitory receptor CTLA-4 is not expressed on resting T cells, but is rapidly induced on T cell activation and acts to modulate the response. Mice deficient in CTLA-4 exhibit severe autoimmunity (34), demonstrating that CD28 activation needs to be limited by CTLA-4 inhibition for the mouse to establish self tolerance. While both receptors recognize the same ligands, CD80 and CD86, the inhibitory CTLA-4 binds with much higher affinity than does CD28, and inhibitory signals override stimulatory signals to limit T cell activation. This has helped to establish the paradigm that inhibition predominates over activation.

In NK cells, the discovery of KIR and Ly-49 activating receptors that recognize the same ligands as their inhibitory counterparts prompted the examination of the relative binding affinities of the inhibitory and activating KIR and Ly-49 receptors. Studies of the KIR receptors to HLA-C demonstrated that the inhibitory receptor p58 bound with higher affinity than did the activating receptor p50, and that a single residue was responsible for the differential binding strength (35, 36). Similarly, the human inhibitory receptor NKG2A displayed higher affinity binding to its ligand HLA-E than did the activating NKG2C (37). In mice, the Ly-49D receptor was shown to bind D^d much less strongly than does the inhibitory receptor Ly-49A (38). All of these results have helped to support a model that NK activating receptors might be of lower affinity than their inhibitory counterparts, and that this weaker recognition strength may help explain how

inhibitory receptors can coexist with and control their activating counterparts to restrain auto-aggression, and that this differential binding affinity may influence how these receptors shape NK function (20, 39). It has also been suggested that Ly-49 activating receptors may have low affinity for mouse class I because these receptors are specific for another ligand, possibly a foreign antigen such as a bacterial or viral class I-like proteins or a peptide derived from a pathogen presented by class Ia or Ib molecules (40).

I have shown that Ly-49 activating receptors exist in the NOD mouse strain that share strong sequence identities and similar patterns of ligand recognition with known inhibitory receptors in the C57BL/6 and BALB/c mouse strains. Ly-49P recognizes H-2D^d strongly and perhaps binds H-2D^k weakly, as has been demonstrated for the C57BL/6 allele of the inhibitory receptor Ly-49A (41, 42), and Ly-49W has strong recognition of D^k and moderate recognition of D^d, as we have also shown for the BALB/c allele of Ly-49G (chapter IV). The strong D^d reactivity of the activator Ly-49P, which is much closer in similarity in the CRD to Ly-49A than is Ly-49D, suggests that Ly-49D's weaker ligand binding may be a characteristic of Ly-49D in particular and not Ly-49 activating receptors in general. Although our studies suggest that the activating Ly-49P receptor binds H-2D^d with higher avidity than does Ly-49D, and perhaps with avidity as high as Ly-49A, direct measurement of binding avidities will necessitate methods such as surface plasmon resonance (BIAcore) which can measure real-time quantitation of protein-protein interactions (43).

In summary, some activating Ly-49 receptors, such as Ly-49P and W, do not appear to have a decreased affinity for class I ligands compared to their inhibitory counterparts. The activating Ly-49D receptor recognizes mouse H-2D^d with low affinity,

but that may be because its preferred ligands are not allogeneic class I. For instance, Ly-49D kills CHO cells efficiently (17), suggesting that it recognizes a non-allogeneic Chinese hamster class I (19) with high affinity. Unlike the CD28/CTLA-4 paradigm, homozygous mice have not demonstrated consistent coexpression of self-specific inhibitory/activating receptor "pairs", suggesting that a mechanism to allow their coexistence may not be necessary.

C. Ly-49 Recognition of Class I Ligands: Genes vs. Alleles

Similarly to the human *KIR* gene cluster, the mouse *Ly-49* gene cluster encodes closely related proteins and appears to be mutable and rapidly evolving. The cloning of *Ly-49* cDNAs from several mouse strains has resulted in the discovery of multiple distinct *Ly-49* coding sequences in different strains with high sequence identity, resulting in uncertainty as to whether these sequences represent distinct genetic loci, or if they are different alleles of the same gene (44). In one instance, the *Ly-49I* gene from the B6 mouse was originally designated as the B6 allele of *Ly-49C* because of its high sequence identity to the *Ly-49C* gene in the BALB/c mouse (45). With human KIR genes, a convention was developed by which similar sequences that possess more than 20 nucleotides differences are probably different genes, while those that differ by less than nine nucleotides are likely alleles (46). When a number of new Ly-49 genes were found in the 129/J mouse, a similar sequence identity convention was followed in the designation of sequences as new genes or alleles of different genes (47).

The *KIR* gene complexes of different individuals have been assigned into *A* haplotypes and *B* haplotypes on the basis of several features, including fewer genes with

less variability in the *A* haplotypes (46). Because of the susceptibility of *KIR* genes to duplicate, delete and recombine, the division in *KIR* haplotypes is indistinct, and it is likely that some haplotypes will combine features of both *A* and *B* haplotypes. Even with the diversity within each haplotype, it is believed that the complexities of differentiating alleles and loci will be resolved by studying *KIR* haplotypes (46).

While the genetics of human NK receptors is especially daunting due to the extreme polymorphism of receptors and tremendous heterogeneity of human individuals, the genetics of mouse NK receptors should be much more approachable due to the availability of inbred mice. The genealogies of inbred mouse strains have been catalogued and described, and strains divided into seven categories based on origins and relationships (48). Recent work proposes the existence of *Nkc* haplotypes based on mouse genealogy, and suggests that there are a limited number of *Ly-49* gene clusters, with an identical (or similar) *Ly-49* gene cluster appearing in several mouse strains that share an *Nkc* haplotype (29).

An apparent contradiction to the *Nkc* haplotypes appears with the analysis of Ly-49 cell surface expression on $DX5^+$ (NK and NKT) cells using monoclonal antibodies, since patterns of Ly-49 expression do not always correspond to the *Nkc* haplotype analysis (30). For instance, *Nkc* haplotype analysis would suggest that CBA/J, DBA/2, AKR, BALB/c, and C3H share a *Ly-49* gene cluster, but Ly-49C/I cell surface expression (as defined by 5E6 mAb staining) is high on BALB/c and DBA/2, moderate to low on CBA/J and C3H, and very low or negative on AKR. These two analyses can still be consistent because *Ly-49* gene expression is moderated by several factors such as class I

MHC expression, and thus the Ly-49 genetic makeup may not always correlate with the Ly-49 cell surface expression.

The *Nkc* haplotype analysis suggests that the *Ly-49* gene regions of the C57BL/6, 129, BALB/c, and NOD mouse strains differ from each other, while the BALB/c and CBA mice may possess the same or similar *Ly-49* gene regions. In support of the existence of *Ly-49* gene cluster haplotypes, cloning of *Ly-49* genes from 129 and BALB/c mice has resulted in the discovery of *Ly-49* genes or gene alleles that are not expressed in the C57BL/6 mouse ((3, 47, 49, 50) and Table 5-1). My examination of the NOD mouse has found new *Ly-49* cDNA sequences encoding alleles for the C57BLB6 genes *Ly-49A, D, and G* as well as the *ly49m* pseudogene, an allele for the 129 *Ly-49P* gene, and a novel gene *Ly-49W* not found in B6, BALB/c or 129 mouse strains (4, 5). In addition, the only *Ly-49* gene reported from the CBA mouse, *Ly-49L*, is identical to the *Ly-49L* gene that I have isolated from BALB/c (5, 14). Similarly, the *Nkc* haplotype analysis suggests that NOD and SWR mice share a *Ly-49* gene cluster, and the only *Ly-49* gene that has been reported from the SWR mouse, *Ly-49A*, is identical to the *Ly-49A* gene that I have found in the NOD mouse (4, 51). Mouse SNP databases have recently become commercially available from Celera Genomics for mouse strains C57BL/6/J, 129X1/SvJ, 129S1/SvlmJ, DBA/2J, and A/J. The *Ly-49* gene regions of the C57BL/6 and 129 mouse strains have been extensively screened (21, 23), but additional information on the allelic differences in the *Ly-49* gene cluster could be obtained from the DBA/2 and A/J mouse strains, which based on the *Nkc* allotype analysis may share a similar or identical *Ly-49* gene cluster with BALB/c. These new SNP databases may help in addressing the question of cross-strain differences in the NKC in general and Ly-49 genetics in particular.

It has been believed that genomic mapping of the *Ly-49* gene cluster in different mouse strains would allow the identification of closely related sequences as different genes or alleles of the same gene, but this assumed that the gene arrangement of the *Ly-49* gene cluster would be constant in different strains of mice. The examination of the Ly-49 gene clusters in the B6 and the 129 mouse strains demonstrates that this assumption was incorrect (21, 23), and the gene organization likely differs between haplotypes as is seen in humans. An inspection of Ly-49 genes isolated from different mouse strains supports the idea that *Ly-49* allotypes may be limited in number, with the same or similar *Ly-49* gene clusters found in genealogically related strains. The defining of genes contained in different *Ly-49* gene cluster allotypes will allow the logical design of experiments to determine how class I phenotype and other traits control the expression and regulation of NK receptors. This has new relevance in the discovery presented in chapter IV of this thesis that different alleles of the same gene exhibit only partially overlapping ligand specificities and binding affinities.

In summary, the gene arrangement and content within the *Ly-49* gene clusters varies in different inbred mouse strains. It may be possible to classify *Ly-49* gene cluster allotypes based on mouse genealogy, and this may facilitate experimental design to establish factors influencing the inheritance, expression and regulation of NK receptors in a defined animal model.

D. Binding of Class I to Ly-49: Site 1 vs. Site 2

Ly-49A recognition can be blocked by an antibody specific for the α1/α2 region of D^d, but not an antibody to the α3 domain (41, 52). Furthermore, a K^b/D^d chimeric

molecule with the α1/α2 domains of K^b and the α3 domain of D^d is not recognized by Ly-49A (4), while the corresponding D^d/K^d chimeric protein is recognized by Ly-49A (53). These results and several others demonstrate the importance of the polymorphic α1/α2 area of D^d in its recognition by Ly-49A.

The co-crystal structure of the CRD of Ly-49A complexed with H-$2D^d$ shows two points of contact on D^d by the Ly-49A homodimer, and these were named site 1 and site 2 (54). Both sites involve the α1/α2 region of D^d. Site 1 is small in area on one end of the class I molecule where the α1 domain ends and the α2 domain begins. The Ly-49A surface fits closely and with high complementarity to the D^d surface at site 1. Ly-49 receptors and class I display a great deal of polymorphism at this putative interaction site, and our own studies are consistent with these residues determining the specificity of Ly-49P and Ly-49W to D^d and D^k, respectively (10). The site 2 interface is a broad concave site situated below the peptide binding platform that includes the D^d α1, α2, α3 and β_2m domains, where both subunits of the Ly-49A homodimer fit in an area that overlaps the CD8 binding site. All seven Ly-49 residues that interact with D^d at site 1 also interact at site 2, while five Ly-49A residues interact at site 2 alone. Site 2 was initially considered less likely than site 1 to have biological significance because the interfacing surfaces of Ly-49A and D^d do not show a very close fit, much of the area of contact is in regions of class I that show limited polymorphism, and the Ly-49A/D^d site 2 interaction would happen more easily between molecules on the same cell than on different cells (55).

One study tested the importance of Ly-49A residues that interact at site 2 exclusively compared with those that interact with both sites 1 and 2. Surface plasmon resonance (BIAcore) measured the binding of mutated Ly-49A proteins to soluble

transgenic wild-type D^d. Three different Ly-49A residues that interact exclusively with site 2 significantly decreased the D^d binding (although mutation of two other Ly-49A residues that bind site 2 resulted in moderate increases), while mutation of Ly-49A residues that bind both site 1 and site 2 showed abrogation of binding with the mutation of two residues and moderate decreases of binding with the other five residues mutated, leading the authors to the conclusion that site 2 is the primary binding site of Ly-49A with H-$2D^d$ (56).

Another study assessed whether D^d proteins mutated at site 1 and site 2 and expressed by C1498 cells could bind Ly-49A tetramers or protect against cytolysis by Ly-$49A^+$ lymphokine-activated killer cells (LAK) (57). Most of the alanine single mutants had little effect on Ly-49A recognition, but three mutations of D^d site 2 residues, R6A, D122A and K243A, showed total elimination of Ly-49A binding or protection from Ly-$49A^+$ LAK killing. These three residues are located in the α1, α2, and α3 domains, respectively, and are conserved in almost every MHC class Ia and class Ib protein. While it is possible that the non-conservative alanine substitutions may have disrupted the configuration so that the expressed proteins are no longer recognizable as class I proteins to the Ly-49A receptor, that situation is unlikely since these are solvent exposed residues and probably do not act to stabilize the class I structure. Aside from that point, Ly-49A interaction with these highly conserved class I residues on D^d does not offer a straightforward explanation for the observed ligand specificities of Ly-49A for D^d and D^k. This study also showed that Ly-49A recognition of D^d was blocked by an antibody to β_2m, thus providing evidence that Ly-49A binding involves interactions with class I

residues in all four regions, α1, α2, α3 and β_2m, but all of these interactions would possible with any class Ia and class Ib protein.

We have provided evidence that the site 1 interface is important in generating ligand specificity in Ly-49 ligand binding. In chapter IV of this thesis, I have shown that the BALB/c and B6 alleles of Ly-49G2 demonstrate different specificities and affinities of ligand binding, where Ly-49G$^{BALB/c}$ binds strongly to D^k and moderately to D^d while in contrast Ly-49G^{B6} has low affinity for D^d and does not recognize D^k at all. Molecular modeling based on the crystal structure of Ly-49A with D^d was employed to try to determine if this one-residue difference could clarify the differential ligand recognition of these two alleles. Of the five residues in the CRD that differ between Ly-49G^{B6} and Ly-49G$^{BALB/c}$, only residue 246 interacts directly with class I and it does so at both sites 1 and 2. Ly-49A and Ly-49G^{B6} have aspartate at position 246 which can form salt bridges with class I residues Lys173 (site 1) and Lys243 (site 2). Ly-49G$^{BALB/c}$ cannot make either salt bridge due to the Asp246→Gly substitution, and at site 2 no close residues can compensate for these stabilizing interactions. Consequently, if Ly-49 Asp246 interaction with Lys243 at site 2 was important for receptor binding, Ly-49G$^{BALB/c}$ would bind D^d and D^k more poorly, but instead both of these ligands are recognized much more strongly. However, at the site 1 interface, the Asp246→Gly substitution can be compensated for by the side-chain rearrangement of Asp244 in Ly-49G$^{BALB/c}$ to form a salt bridge with Arg170 in site 1 of D^d, as we proposed for the interaction of D^k with Ly-49W (5). The Asp246→Gly substitution creates space and enhances backbone flexibility. Both effects can increase the ability of Ly-49G$^{BALB/c}$ to adjust its conformation to best fit potential class I ligands, resulting in a broader class I specificity. Thus, our results are consistent

with site 1 binding being crucial for Ly-49G$^{BALB/c}$ interaction with class I MHC. This study is significant for several reasons. First, the Ly-49 molecules used are not mutants derived by substitution of single residues, but actual wild-type proteins that have evolved their peptide sequences to form functional three-dimensional structures. Second, unlike the mutagenesis studies, the residue altered in the Ly-49G$^{BALB/c}$ sequence not only destroys its ability to form interactions with the site 1 and site 2 partners available to Ly-49G^{B6}, but permits a new Ly-49 interaction with a D^{d} site 1 residue (but with no possible interaction with site 2 residues) to stabilize Ly-49G$^{BALB/c}$ binding to D^{d}.

However, our results must be reconciled with the mutagenesis studies that conclude that site 2 is the functional site in the Ly-49 interaction with D^{d}. Site 1 is small in area, but we have provided evidence that this area may be involved with ligand specificity (chapter IV and (10). Site 2 is large and could form sufficient interactions to stabilize receptor/ligand binding, but the site 2 residues that appear to be essential are also common among all class I molecules (57). One possibility is that site 1 interactions could provide ligand specificity and initial ligand recognition, while stable ligand binding could be dependent on site 2 interactions augmenting the ligand-specific, but tenuous, site 1 interactions.

In conclusion, molecular modeling of the binding of Ly-49G^{B6} and Ly-49G$^{BALB/c}$ to the class I ligands D^{d} and D^{k} based on the Ly-49A/D^{d} co-crystal structure suggests that ligand specificity of Ly-49 receptors with class I occurs at the site 1 interface, and that the site 2 interface may be slightly different than that observed in Ly-49A ligand binding. Confirmation of the role of site 1 in the interaction of Ly-49G$^{BALB/c}$ with H-2D^{k}, and

whether the site 2 interactions differ from that observed in Ly-49A with H-2D^d, will require crystallographic or other structural studies.

E. Future Directions

Can NK cells express Ly-49 activating receptors that strongly recognize self class I?

The ligand specificities for the activating receptors Ly-49D, P and W are now known, and I have not found their transcripts in inbred mouse strains expressing their class I ligands. One possibility is that there is selection pressure against mice that express activating Ly-49 receptors for self class I, resulting in mouse populations without self-reactive Ly-49 activating receptors after several generations of inbreeding. The Ly-49 expression profiles of outbred wild-type mice have not been examined, but these mice would express a wider range of class I molecules and, possibly, a greater number of activating Ly-49 receptors due the greater number of environmental challenges in the wild. It would be interesting to observe if self-specific activating Ly-49 receptors are expressed in these mice and if so, what mechanisms ensure self-tolerance. An approach to addressing this question could begin with well-defined inbred mouse strains.

Cross-bred mice have the ability to express both D^d and its low affinity receptor Ly-49D (15, 16). It has not been demonstrated if a mouse could express activating Ly-49 receptors with high affinity for self MHC proteins. Possible outcomes of that situation could be:

(a) The mouse may have a decreased lifespan due to severe autoimmunity.

(b) To maintain self tolerance, NK cells expressing activating Ly-49 receptors exhibiting strong recognition self-specific activating Ly-49 might be deleted without expansion, possibly by the initiation of apoptosis, to protect the organism from harm. Cross-linking Ly-49D with a monoclonal antibody was found to induce apoptosis-related genes (58), suggesting that activating Ly-49 receptors may induce apoptosis upon strong receptor binding.

(c) Coexpressed inhibitory receptors may always dominate even if the affinity of the inhibitory receptor is less than that of the activating receptor in order to maintain self tolerance. It has been demonstrated that tolerance in the Ly-49D$^+$ subset of NK cells from H-2D^d mice is mediated by co-expression of inhibitory receptors Ly-49A or G (16), even though Ly-49G has low affinity for D^d (chapter IV and (42)). This study also suggests that the level of self-specific inhibitory receptors may be up-regulated to counter the intensity of signals generated from activating receptors by self cell targets.

(d) It has been shown that Ly-49 cell surface expression can rapidly down-regulate in the presence of its class I ligand (59, 60) so self tolerance may be induced by down-regulation of activating receptors to self class I, possibly by the transfer of class I onto the NK cell and receptor/ligand uptake. While the inhibitory receptors Ly-49A and Ly-49G mediated the transfer of target cell D^d to the NK cell and became internalized, Ly-49D did not mediate D^d transfer and did not become internalized, leading to the suggestion that the ITIM somehow helps mediate the class I transfer so that only inhibitory receptors transfer and internalize ligand (61). In this case, the inability of Ly-49D to mediate D^d transfer and internalization may be due to its weak binding to D^d, since Ly-49A mediates

the uptake only of its strong ligands Dd and Dk, and not its weak ligand D^b (62) and H-$2D^d$ has been shown to bind to Ly-49D much more weakly than to Ly-49A (38).

(e) Finally, the activating receptors may regulate cell surface expression by glycosylation. The Ly-49D receptor, which has low affinity for mouse class I molecules, has three potential *N*-linked glycosylation sites while the NOD allele of the Ly-49P receptor, which has a high affinity for mouse class I, has only one potential *N*-linked glycosylation site. In the NOD mouse, the Ly-49D allele has a high expression in both non-activated and IL-2-activated NK cells (unpublished) while the majority of cells appear to have a low expression of Ly-49P (or Ly-49A) as determined by A1 antibody staining, with a small fraction of high expressing cells that may be Ly-$49A^+$ (4).

To examine if mice can express a strong activating receptor to self MHC, NOD mice could be crossed with H-2^d mice, or could be engineered to express a transgenic D^d gene. Another mouse that could be used for these experiments is the NOD-related mouse strain NOR, which expresses Ly-49P and Ly-49W and additionally is resistant to diabetes so disease onset would not influence experimental results. With any of these experimental arrangements, it could be observed whether mice can co-express D^d and the activating receptors Ly-49P and/or W. Would a tolerizing process occur (such as activating receptor down-regulation, inhibitory receptor up-regulation, selection of self tolerant NK cells, or apoptosis of self reactive NK cells), or would these mice have a shortened lifespan?

The Role of Ly-49 Receptors in the NOD Mouse

The NOD mouse strain spontaneously develops the autoimmune disorder insulin-dependent diabetes mellitis (IDDM), and one of the IDDM loci, Idd6, maps onto the distal region of chromosome 6 (63) and appears to co-localize with the NKC region (64). I have demonstrated that full-length transcripts for four Ly-49 activating receptors, Ly-49D, P, W and M, are expressed in the NOD mouse (4, 5), which is more full-length Ly-49 activating receptors than the two found in the B6 mouse Ly-49D and H), and the three found in 129/J strain (Ly-49P, R, and U). The introduction of Idd6 onto a diabetes-resistant C57BL/6 background did not result in significantly increased insulitis (65), so this locus appears to interact with gene products from the NOD background. The examination of the expression of Ly-49 receptors in genealogically related but disease-resistant mice, such as the NOR and SWR strains, may help in establishing whether the activating Ly-49 receptors found in the NOD mouse contribute to its immune dysregulation, possibly by mistaking "normal" self ligands as "pathogenic" self. Ly-49P and W transcripts are expressed in the NOR mouse, but I have not examined NOR NK cells for their cell surface expression.

NOD alleles of Ly-49A and G are also expressed. It is likely that the NOD mouse has subsets of NK cells co-expressing inhibitory/activating "pairs" of receptors, Ly-49A/Ly-49P and Ly-49G/Ly-49W, where both members of the "pairs" may exhibit strong binding to their common ligands, D^d and D^k, respectively. We believe that the Ly-49P and Ly-49W activating receptors can bind strongly to their class I ligands, but the binding of D^d and D^k to inhibitory Ly-49A^{B6}, A^{NOD}, and activating P^{NOD} can be measured by BIAcore, as can their binding to Ly-49G^{B6}, G^{NOD}, and W^{NOD}. Both members of each

"pair" are recognized strongly by the same monoclonal antibodies: A1 recognizes both Ly-49A and Ly-49P, while Cwy-3 and 4D11 recognizes both Ly-49G and Ly-49W. Sorted NK cells could be analyzed by single cell RT-PCR to determine the relative numbers of NK cells expressing either or both activating and inhibitory receptors. NK cells co-expressing activating and inhibitory receptor "pairs" can be tested in killing assays to determine if cytotoxicity outcomes are related to relative expression of activating to inhibitory receptors, or if simultaneous receptor binding may cause other outcomes, such as deletion of these cells by the induction of apoptosis.

When the mAb A1 was used to stain either fresh or IL-2 activated NK cells, there was a large population of $A1^{low}$ cells and a small number of $A1^{hi}$ cells (4). We hypothesize that the $A1^{low}$ cells are Ly-49P$^+$ and the $A1^{hi}$ cells are Ly-49A$^+$ that express different levels of cell surface protein in proportion to their number of potential *N*-linked glycosylation sites (one in Ly-49P vs. three in Ly-49A) and this hypothesis can be tested to see if glycosylation is one way the mouse can adjust the cell surface expression of some Ly-49 proteins, such as high affinity activating receptors.

Can We Design NK Receptors to Improve the Outcome of Hematopoietic Transplants?

Hematopoietic stem cells are progenitors of end stage blood cells, and hematopoietic stem cell transplantation is used to treat various leukemia, lymphoma, and inherited stem cell diseases (66). In the treatment of leukemias, the patient's bone marrow and circulating leukocytes, along with the leukemia cells, are destroyed by irradiation or chemotherapy and are replaced with bone marrow or stem cells from a healthy donor. Besides repopulating the transplant recipient with new hematopoietic

cells, the graft carries donor T cells that are crucial for destroying residual malignant cells in the host, and this is called the graft-versus-leukemia (GVL) effect. Unfortunately, these donor T cells also attack other tissue in any host not completely HLA matched with the donor, causing graft-versus-host disease (GVHD). When transplants are made across the histocompatibility barrier, host rejection by the graft can be prevented by extensive depletion of T cells from the graft, but this depletion results in higher leukemia relapse because of the reduction of the GVL effect.

Recently, a study was done showing the role of NK cells on the treatment of acute myeloid leukemia (AML) (31). They started with an analysis of acute myeloid leukemia patients that had received hematopoietic transplants from HLA haplotype mismatched family donors. The 20 cases where the donors expressed only KIR receptors that did not recognize HLA ligands in the recipients (which would result in the donor NK cells killing recipient cells due to their "missing self" HLA) resulted in transplants that were 100% protected against graft rejection, GVHD and AML relapse. In contrast, the 37 cases where there was no KIR ligand incompatibility in the graft-versus-host direction showed some graft rejection, serious GVHD and a 75% probability of AML relapse at 5 years. To further investigate the role of alloreactive NK cells in hematopoietic transplants, they transplanted bone marrow grafts from F_1 H-$2^{d/b}$ mice into the H-2^b parent. While the donor T cells were tolerant of the donor MHC, the donor NK cell subset that was Ly-49A/G$^+$ but Ly-49C/I killed host cells because they lacked H-2^d class I molecules. Strangely, there was no GVDH and no graft rejection, strongly suggesting that NK cells were killing the host's hematopoietic cells (such as T cells and APCs) over non-hematopoietic tissue (such as epithelial cells). The cytolysis of host cell APCs would

prevent the presentation of host antigens to donor T cells and activation of anti-host CTLs. The lack of NK cell cytolysis of epithelial cells would mean that NK cells would not cause GVDH. The authors then presented evidence in mice that alloreactive NK cells could be used in the pre-transplant "conditioning" of hematopoietic transplant patients to decrease the risk of graft rejection and GVHD when transplanting grafts with greater numbers of T cells, which would decrease the risk of leukemic relapse.

This study suggests a beneficial effect of alloreactive NK cells in hematopoietic transplants. The alloreactive NK cells in this study were those lacking expression of any inhibitory receptors, either KIR or Ly-49, that would recognize the graft recipient class I antigens. However, human expression of both KIR and class I genes is extremely variable, and it may be difficult in every case to find NK cells where no KIR receptors recognize any recipient class I. Would it be possible to use NK cells that are alloreactive because of their expression of activating receptors to recipient class I? The KIR activating receptors that have been found thus far exhibit low affinity ligand binding (46) but I have made chimeric molecules which result in activating receptors that contain the extracellular domain and ligand specificity of alleles of the inhibitory receptor, Ly-49G (chapter IV). It would have to be demonstrated that such engineered activating receptors could function in tandem with the activating receptors that recognize the unknown target cell antigens mediating the NK cell cytolysis of hematopoietic cells. Until the AML study, it had not been suggested that NK cells would target hematopoietic cells over epithelial cells, and thus the receptor/ligand combination that causes this effect is unknown. It is possible that the NCR proteins, which are only expressed on NK cells, may be receptors for ligands only expressed on hematopoietic cells. Much more needs to

be discovered about NK cell recognition of hematopoietic and non-hematopoietic targets before NK cells with "designer" class I receptors can be engineered for use in hematopoietic transplants.

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Table 5-1. Ligands recognized by Ly-49 receptors (as of May 2002).

While other studies examining Ly-49 recognition of target cells from different MHC haplotypes have been described, the ligands listed in this table are from studies for which specific class I ligands were established. Studies in bold are described in this thesis.

	Ly-49 Allele	Ligands Described	Technique Used*	References for Ligand Recognition
Ly-49A	C57BL/6	H-2D^d, D^k, D^b	A, P, T	(41, 52, 67)
Ly-49C	C57BL/6	H-2K^b, D^b, K^d, D^d, D^k	A, T	(42, 67, 68)
Ly-49D	C57BL/6	H-2D^d	R	(9)
Ly-49G	**C57BL/6**	H-2L^d, D^d	A, R	(69, 70) **Chapter IV**
Ly-49G	**BALB/c**	**H-2D^k, D^d**	**R**	**Chapter IV**
Ly-49H	C57BL/6	m157 (MCMV protein)		(27)
Ly-49I	C57BL/6	H-2K^d, D^d	T	(67)
Ly-49P	**NOD**	**H-2D^d**	**R**	**Chapter II**
Ly-49W	**NOD**	**H-2D^k, D^d**	**R**	**Chapter III**

* A antibody blocking of Ly-49 receptor/ligand interaction

P adhesion of Ly-49-expressing cells to immobilized purified class I

T binding of class I tetramers to Ly-49-expressing cells

R cytotoxicity mediated by RNK-16 transformants of Ly-49

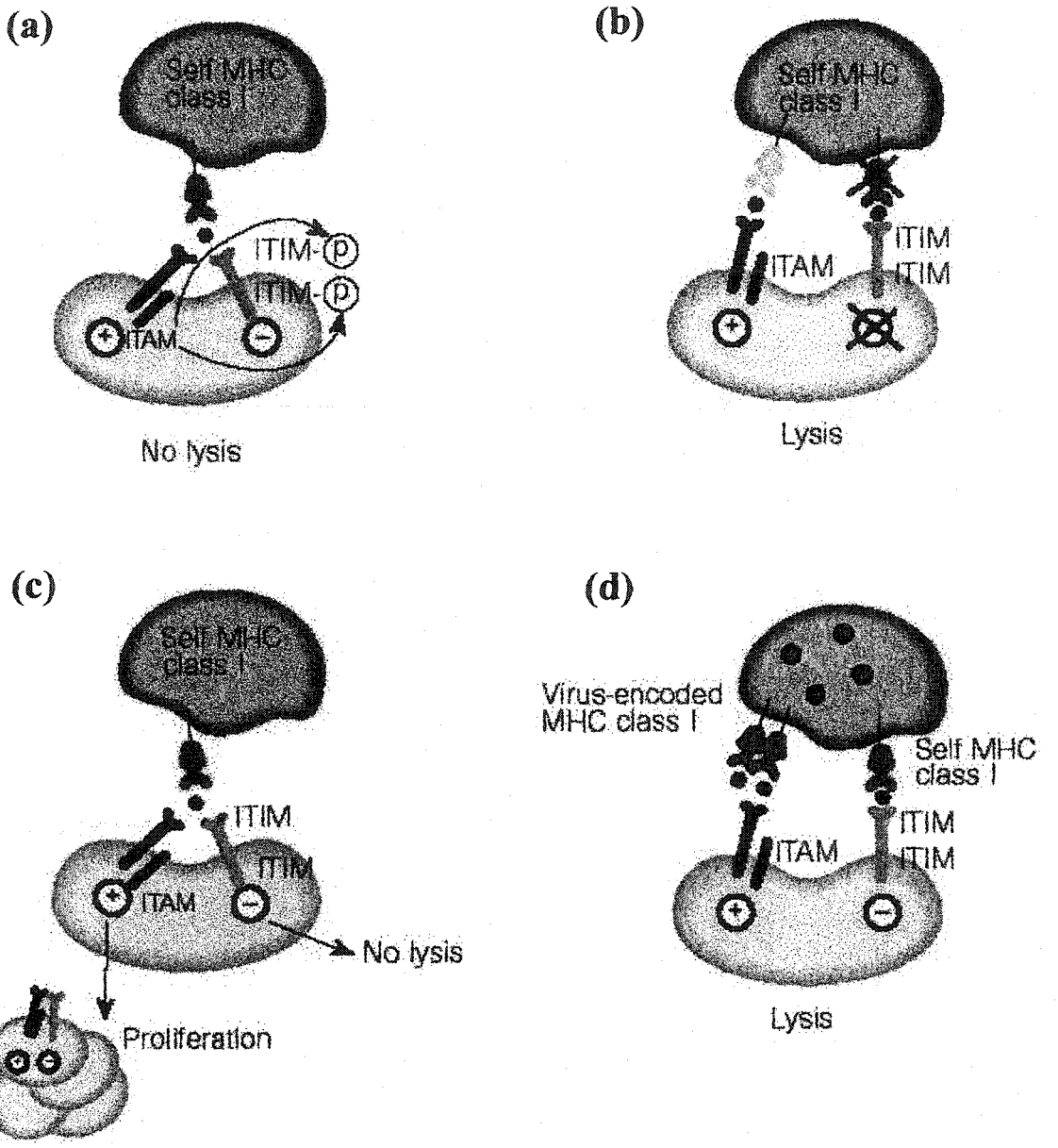

FIGURE 5-1. Hypothetical interactions between activating and inhibitory MHC class I receptors. Inhibitory and activating receptors may recognize the same class I molecules on a target cell. Activating receptors may regulate inhibitory receptors by phosphorylating cytoplasmic ITIMs (a), or may control distinct NK-cell functions. Activating receptors may promote NK-cell proliferation, whereas inhibitory receptors may control effector responses such as cytotoxicity (c). Alternatively, inhibitory and activating receptors may recognize self and non-self class I molecules, respectively (d), or distinct self-class I molecules (b) on the same cell. In this case, activating receptors may allow NK cells to recognize target cells that have selectively lost one class I molecule (b), or target cells that express virally encoded MHC class I molecules/ self class I molecules carrying viral peptides (d). From Colonna, *Nature* 391:642 (1998).