

Heterogeneity Among Ly-49C Natural Killer (NK) Cells: Characterization of Highly Related Receptors with Differing Functions and Expression Patterns

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Summary

Ly-49C is a member of the polymorphic family of murine NK cell inhibitory receptors. The 5E6 antibody that defines a subset of NK cells responsible for the rejection of parental H-2^d bone marrow by F₁ mice has been shown previously to react with Ly-49C. Here, the 5E6 antibody was found to detect two Ly-49C-related molecules in B6 mice. Two cDNA clones were isolated from B6 NK cells, one identical to previously reported Ly-49C^{B6} and the other a novel cDNA. The deduced amino acid sequence of the latter differs from that of Ly-49C^{BALB} at only 4 residues, whereas the previously reported Ly-49C^{B6} differs at 22 residues. Flow cytometric analyses of COS cells transfected with the two cDNAs showed that the 5E6 antibody binds to both Ly-49 molecules, while another anti-Ly-49C antibody, 4LO3311, binds to the newly described Ly-49C but not the previously reported Ly-49C^{B6}. Two-color flow cytometric analysis detected 5E6⁺4LO3311⁻ as well as 5E6⁺4LO3311⁺ subsets of NK cells from B6, but not BALB/c, mice. The level of Ly-49C expression on B6 NK cells detected by the 4LO3311 antibody was substantially lower than that on BALB/c NK cells. Binding specificity of the novel Ly-49C^{B6} was indistinguishable from that of Ly-49C^{BALB}, whereas no binding was detectable with previously reported Ly-49C^{B6}. These results demonstrate that the newly described Ly-49C^{B6}, not the previously reported Ly-49C^{B6}, is the probable B6 allelic form of Ly-49C. The previously reported Ly-49C^{B6} must be encoded by a separate gene and should be renamed Ly-49I. The implication of these results with respect to the role of Ly-49C in hybrid resistance is discussed.

Ly-49C is a member of the Ly-49 family of NK cell inhibitory receptors (1). It was initially identified through gene cloning based on cross-hybridization with the Ly-49A probe (2). The 5E6 antibody, which detects NK subsets responsible for hybrid resistance against H-2^d but not H-2^b bone marrow grafts in F₁ hybrid mice (3), was subsequently found to bind to Ly-49C (4). Flow cytometric analysis of NK cells showed that Ly-49C is expressed on distinct NK subsets (4, 5). In *in vitro* cytotoxicity assays, 5E6⁺ NK cells from (NZB × B6)F₁ mice lyse BALB/c (H-2^d) but not B6 (H-2^b) target cells (5). The resistance of H-2^b target cells to 5E6⁺ F₁ NK cells is reversed by the 5E6 antibody. Therefore, Ly-49C is considered to be an inhibitory NK receptor for class I MHC. The specific interaction between Ly-49C and class I MHC has been demonstrated by the binding of cells to Ly-49C-transfected COS cells and inhibition of the cell binding by specific class I MHC antibodies (4, 6).

Further studies to elucidate the functional role of Ly-49 in the regulation of NK cytotoxicity are complicated by the fact that Ly-49 represents a closely related and highly polymorphic family of molecules. It is currently difficult to determine whether Ly-49 molecules in different strains of mice represent allelic forms of the same loci or whether they are encoded by different genes. Furthermore, allelic forms of Ly-49 molecules may differ from each other in their specificities for class I MHC. These problems are particularly relevant to hybrid resistance. The initial Ly-49C cDNA to be described was isolated from a (B6 × CBA)F₁ mouse cDNA library (2). The identical gene was found to be expressed in BALB/c mice. A related gene, isolated from B6 mice, differs from Ly-49C^{BALB} in 22 amino acid residues but has been considered to be the allelic form (7). Both Ly-49C^{BALB} and Ly-49C^{B6} are detected by the 5E6 antibody. In *in vitro* cytotoxicity assays, 5E6⁺ NK cells

from B6 mice are able to kill H-2^d, but not H-2^b, target cells, while those from BALB/c mice kill neither H-2^d nor H-2^b targets (5). This difference is thought to be due to different ligand specificities of the two putatively allelic forms of Ly-49C. It is unknown why 5E6⁺ NK cells of F₁ mice that express either or both of the two allelic forms of Ly-49C fail to receive negative signals from parental H-2^d bone marrow cells.

In this study, we further characterize Ly-49C from B6 and BALB/c mice by cDNA cloning as well as with a newly available Ly-49 antibody. Our results demonstrate that the 5E6 antibody that has been used to identify Ly-49C in fact recognizes two distinct Ly-49C-related receptors in B6 mice and that the two receptors are differentially expressed on B6 NK cell subsets. One of the two B6 Ly-49 molecules identical to previously reported B6 Ly-49C shows no specific binding to H-2^d or H-2^b targets, whereas the second B6 Ly-49C molecule binds to class I MHC in a manner indistinguishable from the BALB/c receptor. Based upon sequence homology, the latter receptor is most likely the B6 allele Ly-49C, whereas the former must be the product of another gene highly related to Ly-49C. Therefore, these findings call for a reevaluation of previous studies that have equated 5E6⁺ NK cells with Ly-49C, particularly with regard to the role of the Ly-49C receptor in hybrid resistance (5), as well as its potential allelic exclusion (7, 8).

Materials and Methods

Antibodies and Flow Cytometry. The 4LO3311 antibody has been described (9). The 5E6 hybridoma was provided by Dr. V. Kumar (University of Texas Southwestern Medical Center, Dallas, TX). Flow cytometric analysis of transfected COS were carried out as described (4). NK cell staining procedures were as follows: splenic NK cells (3–5 × 10⁵) were first incubated with unlabeled anti-FcR Ab to block nonspecific binding of anti-Ly-49C antibodies. This Ab was purified from the supernatant of the 2.4G2 hybridoma line purchased from the American Type Culture Collection (Rockville, MD). NK cells were then stained with FITC-conjugated 5E6, biotinylated 4LO3311, or both Ab added in this sequence. SA-PE conjugate was used for the detection of the red fluorescence in one color analysis. SA-RED670TM conjugate was used for two-color analysis to avoid reduction of the green fluorescence signal by electronic compensation. Stained cells were washed twice in PBS containing 1% BSA and 0.2% sodium azide. NK cell analysis based on collection of 10,000 events per sample was performed on a Coulter Epics[®]XL-MLC (Coulter Electronics, Hialeah, FL) equipped with a 488 nm argon laser. Data analysis was done with XL software (Coulter Electronics).

Isolation of NK Cells. IL-2-activated NK cells used for cDNA cloning were prepared as described (4). Splenic NK cells were isolated by nylon wool column followed by depletion of CD4⁺ and CD8⁺ cells as previously described (10). Flow cytometric analysis of the isolated NK populations showed 64–83% of these cells from B6 mice were NK1.1⁺.

Ly-49C PCR. PCR primers specific for Ly-49C were designed corresponding to sequences located in the 5' and 3' untranslated regions of the Ly-49C^{BALB} transcript. The sequences of the oligonucleotides were 5'-AGTACCGCGGAACGCCAA-

CGTTTCAGA-3' and 5'-TACTCCGCGGGAGACTATGT-TCTGTAA-3'. The first strand of cDNA was generated in a 25 μl mixture containing 10 μg total RNA (isolated from B6 NK cells cultured for 10 d in 1,000 U of IL-2), 0.5 mM each of four deoxyribonucleotide (triphosphate), 4 μg/ml random hexamers, 10 mM dithiothreitol, and 200 U of reverse transcriptase. The reaction was incubated at 37°C for 1 h followed by 5 min at 95°C to inactivate the enzyme. 1 μl of this reaction was then subjected to PCR using Elongase (Canadian Life Technologies, Burlington, Ontario) according to the protocols provided by the manufacturer. 35 cycles were carried out as follows: 1 min denaturation at 94°C; 1 min annealing at 45°C; and 3 min extension at 72°C. This was followed by a final extension for 5 min at 72°C. PCR products of ~1 kb in size were purified from a 0.8% agarose gel, cut with SstII to remove the primer tails, and subcloned into the SstII site of pBluescript-KS⁺. All PCR-derived clones described in this paper were obtained in at least two independent PCR and subcloning experiments and were sequenced in their entirety.

Transfections and Adhesion Assays. The Ly-49A^{BALB} cDNA was isolated by PCR as described by Held et al. (8). The Ly-49E^{B6} and Ly-49F^{B6} cDNAs (11) were obtained from Dr. W. Yokoyama (Washington University, St. Louis, MO), and the Ly-49G^{BALB} cDNA was provided by Dr. S. Anderson (NCI, Frederick, MD). Transfection of COS cells with Ly-49 cDNA and cell adhesion to the transfected COS cells were carried out as described (4).

Results

Isolation of Ly-49C cDNAs from BALB/c and C57BL/6 Mice. Southern blot analysis of genomic DNA of B6 mice using a single exon probe derived from the 3' end of the Ly-49C cDNA revealed 4–5 hybridizing bands (data not shown). Under the conditions used, the probe cross-hybridized with Ly-49H cDNA but not other Ly-49 cDNAs

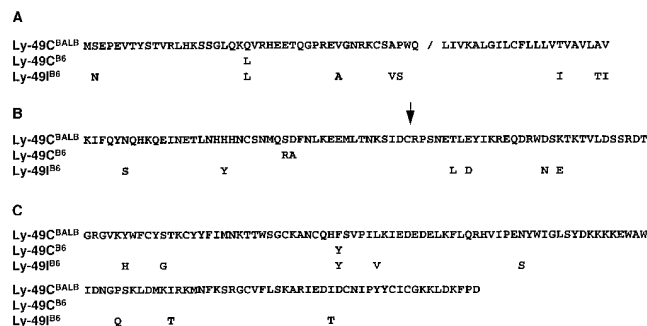


Figure 1. (A) Cytoplasmic/transmembrane domain; (B) Stalk; (C) Carbohydrate recognition domain (CRD). Deduced amino acid sequences of two forms of Ly-49C from B6 NK cells. The amino acid sequences of Ly-49C^{B6} and Ly-49I^{B6} are aligned with that of Ly-49C from BALB/c mice. The sequences are divided into those in the cytoplasmic/transmembrane, stalk, and CRD regions. The arrow indicates the beginning of the region within the stalk region that previously has been shown to be important for the binding of Ly-49 to class I MHC (6). For the sequences of Ly-49C and I from B6 mice, only the residues different from those of BALB/c mice are shown. The CRD of Ly-49C was deduced from the previous studies on genomic clones of Ly-49, which indicates that the CRD is encoded by three exons (2). The nucleotide sequence of the Ly-49C^{B6} cDNA is identical to an unpublished Ly-49C^{B6} cDNA recently deposited in Genbank by Sentman and coworkers (these sequence data are available from EMBL/Genbank/DDBJ under accession number U56404).

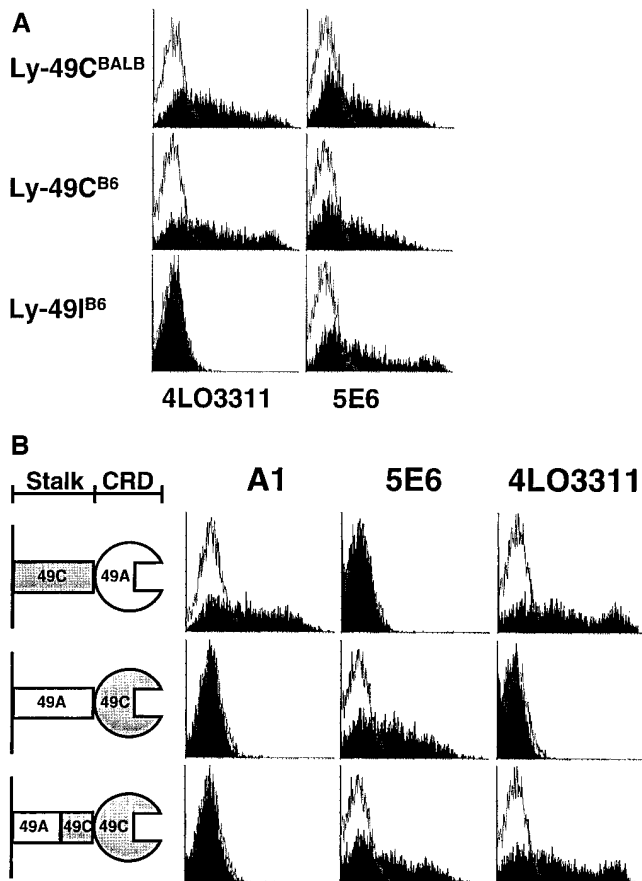


Figure 2. Specificities of anti-Ly-49C antibodies. (A) Ly-49C^{B6} and Ly-49I^{B6} were expressed on COS cells and stained with 5E6 and 4LO3311. Solid histograms represent cells transfected with Ly-49C and empty histograms are cells transfected with vector alone. (B) COS cells were transfected with chimeric Ly-49A/C as indicated and stained with A1 (*anti-Ly-49A*), 5E6, or 4LO3311. Solid histograms represent cells transfected with chimeric Ly-49 and empty histograms are cells transfected with vector alone.

identified thus far (Ly-49 A, B, D, E, F, and G). Each band likely represents an individual gene (or possibly pseudogenes), because sites for the restriction enzymes used for the analysis are not found within the single exon probe. To identify Ly-49C-related genes in B6 mice, PCR was performed on cDNA prepared from IL-2-activated NK cells from B6 mice. The PCR primers used in these experiments were designed so as to amplify Ly-49C and none of the other known members of the Ly-49 family. A total of eight clones were generated and fully sequenced. One of them was found to be identical to Ly-49C^{B6} previously reported (7), and the other seven all encoded a novel cDNA highly related to Ly-49C^{BALB}. The predicted protein encoded by the novel B6 cDNA differs from the Ly-49C^{BALB} by only 4 amino acids, whereas the previously reported Ly-49C^{B6} differs at 22 amino acid residues (Fig. 1). At the nucleotide level, the coding region of the novel cDNA is 99.3% identical to that of BALB/c Ly-49C cDNA, whereas the previously reported cDNA sequence is 95.9% identical to the BALB/c form. For clarity, the previously reported Ly-49C^{B6} has

been renamed Ly-49I^{B6} and the newly described cDNA designated Ly-49C^{B6}.

Specificities of Anti-Ly-49C Antibodies. The 4LO3311 antibody was previously reported to detect the NK cell antigen NK2.1 (9, 12). The specificity of this antibody for Ly-49 was tested by the binding to COS cells transfected with individual Ly-49 cDNAs. Flow cytometric analysis of the transfected COS cells showed that 4LO3311, like 5E6, recognizes Ly-49C^{BALB} but not Ly-49A^{B6}, Ly-49A^{BALB}, B^{BALB}, D^{B6}, E^{B6}, F^{B6}, G^{B6}, G^{BALB}, or H (Gosselin, P., Y. Lusignan, J. Brennan, F. Takei, D. Mager, and S. Lemieux, manuscript submitted for publication). When the two B6 Ly-49C-related molecules were tested, 4LO3311 bound only to the novel Ly-49C^{B6}, while 5E6 reacted with both B6 Ly-49C and Ly-49I (previously reported Ly-49C^{B6}) (Fig. 2 A). Therefore, according to this serologic reactivity, Ly-49C^{BALB} and Ly-49C^{B6} are 5E6⁺4LO3311⁺, and Ly-49I^{B6} is 5E6⁺4LO3311⁻.

As a tool to identify the domains recognized by the 4LO3311 antibody, chimeric Ly-49C^{BALB}/Ly-49A^{B6} cDNA constructs (6) were expressed in COS cells and tested for their antibody reactivity. 5E6 has previously been found to bind epitopes contained within the carbohydrate recognition domain (CRD)¹ (6). However, 4LO3311, was shown to recognize an epitope located in a 32-amino acid segment of the stalk region immediately adjacent to the CRD (Fig. 2 B). This region has previously been shown to be important for specific binding of Ly-49C to class I MHC. In light of the 4 amino acid differences between Ly-49C^{BALB} and Ly-49I^{B6} in this region (see Fig. 1), combined with 4LO3311 reactivity with the former but not the latter, it follows that one or more of these four amino acid residues contribute to the epitope recognized by 4LO3311. It is also possible that the lack of an NH₂-linked glycosylation site in this region of Ly-49I^{B6} affects the binding of the 4LO3311 antibody.

Ly-49C⁺ NK Cell Subsets. The expression of Ly-49C on NK cells from BALB/c and B6 mice was examined by flow cytometry using the 5E6 and 4LO3311 antibodies. The 4LO3311 antibody stained significant fractions of NK cell populations from B6 as well as BALB/c mice (Fig. 3 A), but there were significant differences between BALB/c and B6 NK cells. The staining of BALB/c NK cells with the 4LO3311 antibody was ~10-fold more intense than that of B6 NK cells, and two times more positive cells were detected in BALB/c mice.

Two-color analysis of NK-enriched spleen cells showed that BALB/c mice possess only NK cells that are either double-positive or double-negative for the 5E6 and 4LO3311 antibodies. In contrast, the B6 NK population consists of double negative, double positive, and single positive (5E6⁺4LO3311⁻) subsets (Fig. 3 B). Based on the specificities of the 5E6 and 4LO3311 antibodies described above, these flow cytometric analyses indicate that the two B6 Ly-49C-related molecules are expressed on distinct but possibly

¹Abbreviation used in this paper: CRD, carbohydrate recognition domain.

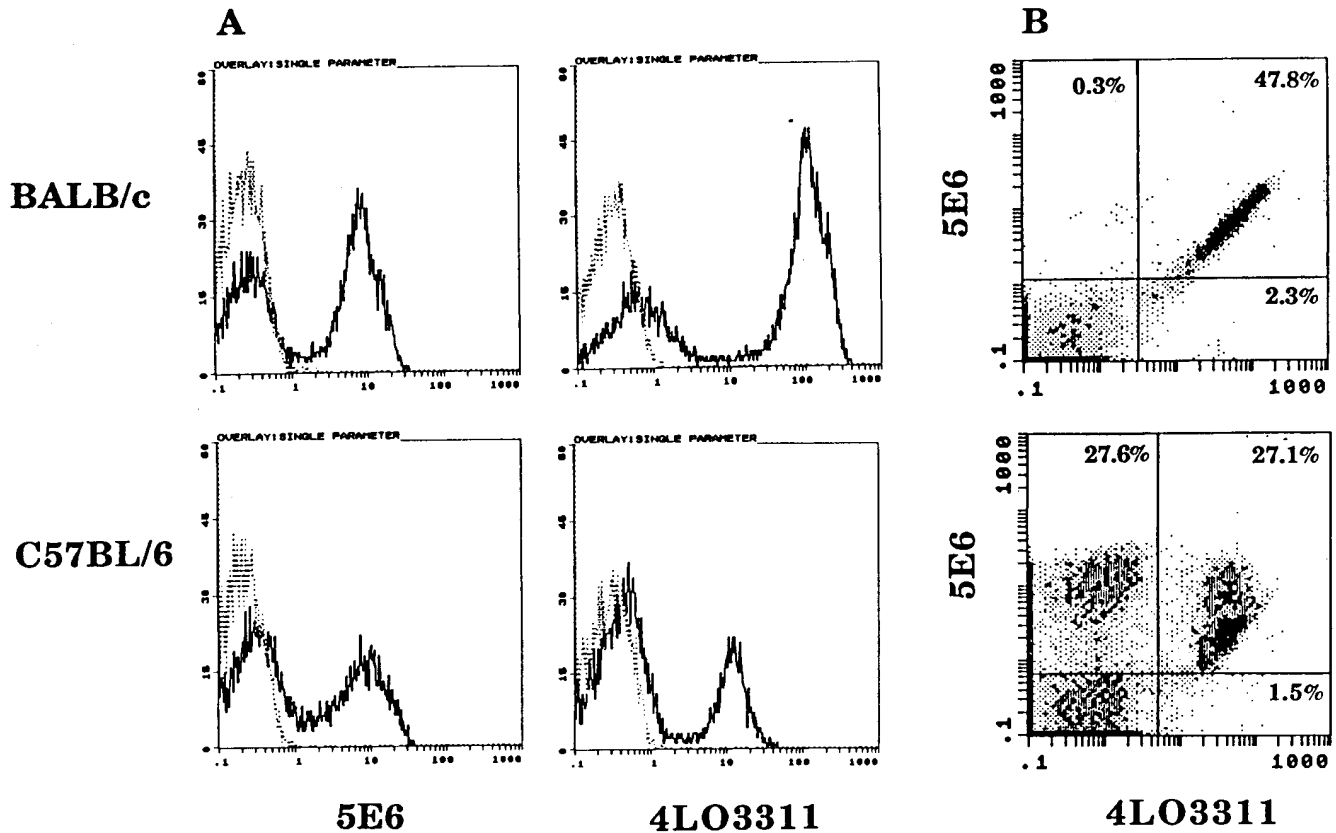


Figure 3. Expression of Ly-49C on NK cells from BALB/c and B6 mice. (A) Splenic NK cells were first incubated with unlabeled anti-Fc γ R (2.4G2) to block nonspecific binding of antibodies then stained with FITC-conjugated 5E6 or biotin-conjugated 4LO3311 and SA-PE. The x and y axes correspond to the log fluorescence intensity and cell count, respectively. Solid histogram shows anti-Ly-49C staining and dotted histogram shows autofluorescence of unstained cells. No difference was observed between unlabeled cells and cells incubated with FITC- or biotin-conjugated isotype-control mAbs (data not shown). (B) NK cells were stained for two-color analysis as described for A, except that SA-RED670TM conjugate was used for the detection of the red fluorescence. The electronic compensations for the two-color analysis were set on samples incubated with each antibody alone.

overlapping subsets of NK cells, while no such heterogeneity is observed with BALB/c NK cells. It should be noted that the double-positive subset of B6 NK cells seems to contain at least two populations differing in the intensities of 5E6, but not 4LO3311, staining. They may represent cells expressing Ly-49C alone and those coexpressing Ly-49C and Ly-49I. It is also possible that these two antibodies may detect other Ly-49 molecules yet to be described on the double positive population.

Recognition of Class I MHC by B6 and BALB/c Ly-49C Receptors. 5E6⁺ NK cells have been thought to receive negative signals upon interactions of Ly-49C with H-2^b and, under certain circumstances, H-2^d antigens on target cells. After having shown that 5E6⁺ NK cells in B6 mice actually comprise two distinct NK cell subsets and that each of these express distinct but highly related receptors, we next investigated the class I MHC binding properties of each of these B6 receptors. It has been shown previously that Ly-49A^{B6} and Ly-49C^{BALB} expressed on COS cells by cDNA transfection mediate cell-cell adhesion by binding to class I MHC antigens on various opposing cell lines (6). Ly-49A^{B6} has been shown to bind H-2^d structures (but not H-2^b), whereas Ly-49C^{BALB} binds to both H-2^d and H-2^b.

When this same assay was used to test the B6 Ly-49 receptors, Ly-49C^{B6} bound to both H-2^d and H-2^b, as was the case for the highly related Ly-49C^{BALB} (Fig. 4 A). Interestingly, Ly-49I^{B6} did not show any class I binding ability in this assay system. The binding was almost completely inhibited by the 4LO3311 antibody (Fig. 4 B), further indicating that the cell adhesion in this system is mediated by Ly-49C.

Discussion

The results presented in this report have demonstrated that two Ly-49C-related molecules are expressed on NK cells from B6 mice. Although Ly-49C⁺ NK cells in B6 mice were previously identified by the 5E6 antibody, our current data have shown that this NK subset is, in fact, heterogeneous and can be divided into subpopulations based on the expression of the two Ly-49 molecules. One of the two Ly-49 molecules described in this study, Ly-49I^{B6}, is identical to the previously reported Ly-49C^{B6} that was considered to be allelic to Ly-49C^{BALB} (7). However, the deduced amino acid sequence of the other Ly-49 is much closer to

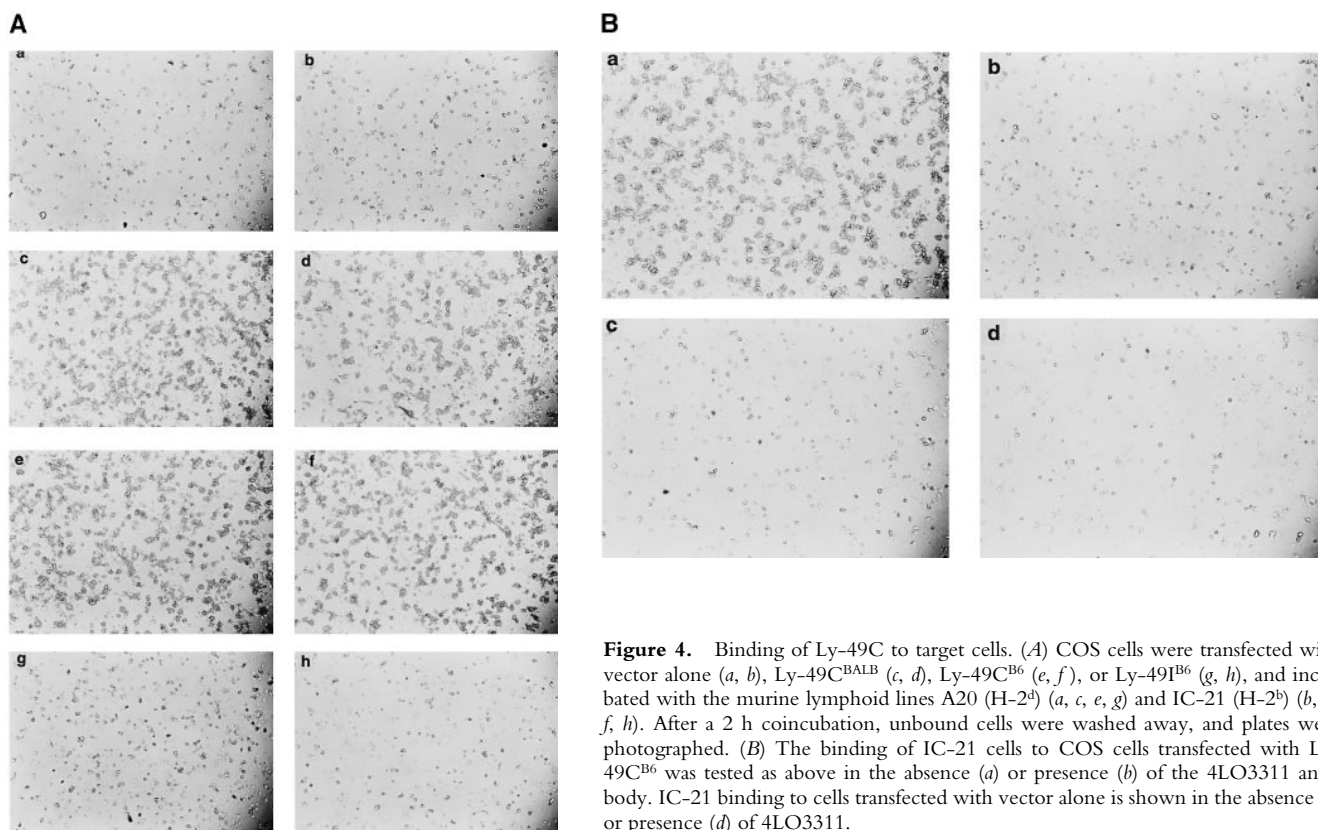


Figure 4. Binding of Ly-49C to target cells. (A) COS cells were transfected with vector alone (a, b), Ly-49C^{BALB} (c, d), Ly-49C^{B6} (e, f), or Ly-49I^{B6} (g, h), and incubated with the murine lymphoid lines A20 (H-2^d) (a, c, e, g) and IC-21 (H-2^b) (b, d, f, h). After a 2 h coinubation, unbound cells were washed away, and plates were photographed. (B) The binding of IC-21 cells to COS cells transfected with Ly-49C^{B6} was tested as above in the absence (a) or presence (b) of the 4LO3311 antibody. IC-21 binding to cells transfected with vector alone is shown in the absence (c) or presence (d) of 4LO3311.

the Ly-49C^{BALB} sequence than that of Ly-49I (4 residue difference versus 22 residue difference). More importantly, this newly described Ly-49C^{B6} shows the same binding specificity for class I MHC as that of Ly-49C^{BALB}, whereas no binding of Ly-49I to those class I MHC is detectable in the cell binding assays in this study. Therefore, the newly described Ly-49C^{B6} is more likely the B6 allelic form of Ly-49C. The previously reported Ly-49C^{B6} must be encoded by a different but highly related gene and has been renamed Ly-49I^{B6}.

Based on our finding that the 5E6 antibody binds to two different Ly-49 receptors, Ly-49C^{B6} and Ly-49I^{B6}, on B6 NK cells and that these receptors differ in terms of their class I recognition abilities and expression on NK cell subsets, our previous understanding of 5E6⁺ NK cells is considered both simplistic and inaccurate. The 5E6 antigen was originally defined as a marker of an NK cell subset in F₁ hybrid mice that rejects parental H-2^d but not H-2^b bone marrow grafts (3). The subsequent identification of 5E6 as Ly-49C and the modulation of NK cell killing in vitro with anti-5E6-Ab has served as strong support that this molecule functions as an inhibitory receptor for class I MHC (5). The role of Ly-49C in hybrid resistance has been complicated by the finding that 5E6⁺ NK subsets from B6 and BALB/c mice differ from each other in their target specificities. The former kill H-2^d, but not H-2^b, targets, whereas the latter do not effectively kill either target (5). To reconcile these in vitro results with the in vivo observation of hybrid resistance, it was proposed that the two

allelic forms of Ly-49C, having significantly different amino acid sequences, may differ in their specificities for class I MHC and that Ly-49C^{B6} may be preferentially expressed on F₁ NK cells, possibly by allelic exclusion (5, 8). However, our present results indicate that Ly-49C^{BALB} and the likely B6 allelic form, Ly-49C^{B6}, have the same binding specificities, whereas Ly-49I^{B6} does not bind to H-2^d or H-2^b target cells. Why, then, do NK cells from F₁ mice fail to receive inhibitory signals from H-2^d target cells? Perhaps, this question can be answered by the receptor calibration model (13–15). According to this model, the expression levels of individual Ly-49 molecules are downregulated by self-class I MHC that interact with Ly-49. Furthermore, inhibition of NK cytotoxicity by Ly-49 is dependent on its level of expression, which is controlled by the affinity of interaction between Ly-49 and particular self-MHC. We have demonstrated that the level of Ly-49C on BALB/c NK cells is nearly 10-fold higher than the level of Ly-49C^{B6} on B6 NK cells as determined by the staining with the 4LO3311 antibody (Fig. 3 A). Based on the receptor calibration model, it is likely that the affinity of the interaction between Ly-49C and H-2^d is lower than that between Ly-49C and H-2^b. Thus, Ly-49C⁺ BALB/c NK cells can be inhibited by H-2^d despite the low affinity interaction due to the high level of Ly-49C expression. In contrast, Ly-49C⁺ NK cells from B6 mice, and likely from F₁ mice as well, fail to receive inhibitory signals from H-2^d because of the low Ly-49C expression and the low affinity interaction, but are inhibited by H-2^b due to its relatively high af-

finity interaction. Recent findings that the expression level of Ly-49C is indeed regulated by class I MHC of the host (Gosselin, P., Y. Lusignan, J. Brennan, F. Takei, D. Mager, and S. Lemieux, manuscript submitted for publication) further supports this model.

It is of interest that the 4LO3311 antibody binds to the stalk region of Ly-49C and yet it effectively inhibits the binding of target cells to Ly-49C-transfected COS cells. On the other hand, 5E6 binds to the CRD and also inhibits the binding of class I MHC to Ly-49C. Previous studies of Ly-49C have found sequences in both the CRD and in the stalk region immediately adjacent to the CRD to be important in binding to class I MHC (6). The results with 4LO3311 further support the hypothesis that the recogni-

tion of class I MHC involves both the CRD and the stalk region of Ly-49C.

These results have demonstrated the complexity of the NK cell receptor Ly-49. The unexpected cross-reactivity of the 5E6 antibody exemplifies the potential problem associated with a highly polymorphic and complex family of molecules such as Ly-49. Until all the Ly-49 genes and the proteins are identified, precise specificities of existing Ly-49 antibodies require careful examination. At the same time, our results suggest a relatively simple explanation of the question of how hybrid resistance is regulated by Ly-49. The finding that the previously reported Ly-49C^{B6} is unlikely to be the B6 allelic form of Ly-49C also calls for a re-evaluation of allelic exclusion of Ly-49C (8).

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