MHC-dependent shaping of the inhibitory Ly49 receptor repertoire on NK cells: evidence for a regulated sequential model

Thomas Hanke[1], Hisao Takizawa[2] and David H. Raulet[3]

[1] Institute for Virology and Immunobiology, University of Würzburg, Würzburg, Germany
[2] Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan
[3] Department of Molecular and Cell Biology and Cancer Research Laboratory, University of California, Berkeley, USA

Engagement of MHC class I-specific inhibitory receptors regulates natural killer (NK) cell development and function. Using both new and previously characterized anti-Ly49 monoclonal antibodies, we comprehensively determined expression and co-expression frequencies of four Ly49 receptors by NK cells from MHC-congenic, MHC class I-deficient, and Ly49A-transgenic mice to study mechanisms that shape the inhibitory Ly49 repertoire. All Ly49 receptors were expressed on partially overlapping subsets. Significantly, in the absence of class I MHC, several receptor pairs were co-expressed more frequently than predicted from a purely random expression model, indicating that biases independent of MHC class I underlie receptor co-expression in some cases. MHC interactions were found to inhibit Ly49 co-expression variably depending on the MHC allele and the receptor pair examined. These data extend previous evidence that interactions with MHC shape the repertoire. It was previously proposed that developing NK cells express Ly49 receptors sequentially and cumulatively, until self-MHC specific receptors are expressed and inhibit new receptor expression. Fulfilling a major prediction of this model, we found that class I recognition by a Ly49A transgene expressed by all developing NK cells equivalently inhibited expression of endogenous self-specific and nonself-specific Ly49 receptors.

Received	30/7/01
Accepted	12/9/01

Key words: Ly49 / NK cell / MHC class I / NK repertoire / Inhibitory receptor

1 Introduction

Engagement of MHC class I-specific cell surface receptors regulates development and function of NK cells [1, 2]. MHC class I-specific receptors either associate with adapter molecules to activate NK cells or utilize an intracellular immune receptor tyrosine-based inhibitory motif (ITIM) to inhibit NK cells [3]. Little is known about the physiological role of activating MHC receptors on NK cells [4]. Inhibitory MHC-specific receptors contribute to the induction and/or maintenance of NK cell tolerance toward cells expressing self-MHC, and allow NK cells to attack cells that down-regulate MHC class I molecules as a consequence of transformation, infection or mutation [5].

Inhibitory CD94/NKG2 heterodimers recognize the nonclassical antigens HLA-E (in humans) or Qa-1 (in mice) which present peptides from the signal sequence of classical (class Ia) MHC molecules [6]. Class Ia-specific inhibitory receptors belong to the Ig superfamily (IgSF), ("KIR" receptors in humans) or to the lectin-like Ly49 family (in mice). In B6 mice, the Ly49 family consists of at least ten members (Ly49A-J) [7, 8]. Eight of them (Ly49A, -B- C, -E, -F, -G2, -I, -J) contain an ITIM. Inhibition of NK cell cytotoxicity by MHC engagement on target cells has been demonstrated for Ly49A [9], Ly49C/I [10] and Ly49G2 [11].

Class Ia-specific inhibitory NK cell receptors discriminate MHC alleles. The functional interaction of Ly49A with D^d has been extensively studied [12]. Besides D^d, D^k as well as H-$2^{f,q,r,s,v}$ molecules serve as functional ligands for Ly49A, while Ly49C specifically interacts with H-$2^{b,d,k,f,q,r,s,v}$ gene products and Ly49I binds to H-$2^{b,d,k,q,r,s,v}$, but not H-2^f molecules [7, 13]. Ly49G2 and Ly49F interact preferentially, if not exclusively with H-2^d [11, 13, 14]. No MHC reactivity has been reported for Ly49B, -E, or -J.

Ly49 and KIR receptors are expressed in a variegated fashion on subsets of NK cells [1, 15, 16]. Analysis of

[I 22286]

Abbreviation: ITIM: Immune receptor tyrosine-based inhibitory motif

0014-2980/01/1111-3370$17.50+.50/0

Ly49 co-expression revealed extensive overlap of NK cell subsets expressing individual Ly49 family members [17, 18]. However, comprehensive studies have been hampered by a limited number of applicable Ly49-specific mAb. As a rough approximation, the fraction of NK cells expressing two Ly49 receptors equals the product of the individual frequencies (the "product rule" [15]), suggesting significant independence in the expression of different Ly49 receptors. Furthermore, most NK cells that express a given Ly49 receptor express only one or the other allele of the corresponding gene, with a smaller subset co-expressing both alleles [19–22]. The data suggest a stochastic mechanism that initiates expression of individual Ly49 alleles/genes [20, 21]. Once initiated, Ly49 expression by NK cells appears to be stable *in vivo* and *in vitro* [9, 20, 23]. Recent data indicate that acquisition of inhibitory Ly49 receptor is cumulative during NK cell development [23–25].

Ly49 expression is also influenced by MHC class I-dependent education processes. In the absence of MHC class I, *i. e.* in β2m-deficient, TAP-1 deficient or β2m/TAP-1 double-deficient mice, the frequencies of NK cells that co-express different inhibitory Ly49 receptors are increased [17, 26, 27]. These findings imply that MHC class I interactions shape the repertoire by restraining the extent of inhibitory receptor co-expression. For example, NK cells co-expressing Ly49A and Ly49G2 are less frequent in H-2^d mice, which express the D^d ligand recognized by both receptors, than in H-2^b mice, which do not contain strong ligands for these receptors [17]. Furthermore, in H-2^d mice engineered to express a Ly49A transgene in all NK cells, 2–3 times fewer NK cells expressed Ly49G2 than in transgenic H-2^b mice. Conversely, Ly49G2 transgene expression in H-2^d mice resulted in a 2–3-fold reduction in the number of Ly49A$^+$ NK cells compared to H-2^b-transgenic mice [28, 29]. These data suggest that cognate MHC recognition limits the generation of NK cells that express an abundance of self MHC-specific inhibitory Ly49 receptors [2, 15].

Two mechanistic models for MHC-specific formation of the NK repertoire have been proposed (reviewed in [2, 15, 30]). In a selection model, developing NK cells express an initially random set of inhibitory Ly49 receptors and are subjected to selection favoring cells expressing "at least one" (to induce tolerance) and "not too many" (to increase usefulness) self-specific receptors. The alternative regulated sequential model proposes that developing NK cells initiate expression of Ly49 receptor genes in a sequential and cumulative fashion, and are tested at each step for expression of "at least one" self-specific receptor based on interactions with MHC molecules in the host. Expression of additional (either self- or non-self-specific) receptors ceases when sufficient inhibitory receptors are engaged. To date, the limited experimental evidence does not allow discrimination of these models.

Here, we took advantage of new Ly49-specific mAb, MHC class I-deficient mice, and a panel of MHC-congenic strains to comprehensively study the effect of MHC on the NK repertoire. We provide evidence for MHC-dependent and MHC-independent factors influencing co-expression of inhibitory Ly49 molecules, and employ a transgenic mouse model to test the regulated sequential model of MHC specific NK cell education.

2 Results

2.1 MHC-shaped co-expression of Ly49A, Ly49G2, Ly49F and Ly49I

We employed recently developed mAb with specificity for Ly49F (HBF) and Ly49I (YLI) together with Ly49A-specific mAb JR9-318 and Ly49G2-specific mAb 4D11 to study the inhibitory Ly49 receptor repertoire on *ex vivo* isolated splenic NK cells (phenotype NK1.1$^+$CD3$^-$ or NK1.1$^+$CD3$^-$CD8$^-$). As shown representatively in Fig. 1 for B10 mice, any combination of Ly49A, -F, -G2 and -I expression was readily detectable on the surface of NK cells. For each of the six pairs of the four Ly49 receptors analyzed, NK cells expressed either none, one, or both of the individual receptors. These data extend earlier results showing that Ly49 receptors are expressed on the cell surface in a variegated fashion and that cell surface co-expression of any receptor pair is allowable. The fraction of NK cells expressing two receptors in combination approximated the multiplied frequencies for the individual receptors (*i. e.* the "product rule" value), indicating that Ly49A, -F, -G2 and -I can be expressed independently of each other on NK cells.

To analyze the effect of MHC on the Ly49 repertoire, we compared the frequencies of Ly49A, -F, -G2 and -I expressing NK cells from a panel of eight MHC congenic B10 mice with that of class I-deficient β2m$^{-/-}$ B6 mice (Fig. 2). With the exception of Ly49I in H-2^r and H-2^f mice, and Ly49G2 in H-2^q mice, Ly49A, -F, -G2 or -I expression was up to 4-fold less frequent on NK cells from class I$^+$ mice (*i. e.* in 29/32 instances) than from class I$^-$ mice. The effect of MHC I was generally more pronounced for Ly49A and Ly49F and less pronounced for Ly49G2 and Ly49I. The data show that most, if not all, class I alleles impact the NK repertoire. Previous analyses have focused on Ly49A, Ly49G2, and Ly49C/I (as recognized by mAb 5E6) expression in class I$^-$ and class I$^+$ H-2^b, H-2^d and H-2^k mice [17, 26]. Our data extend the

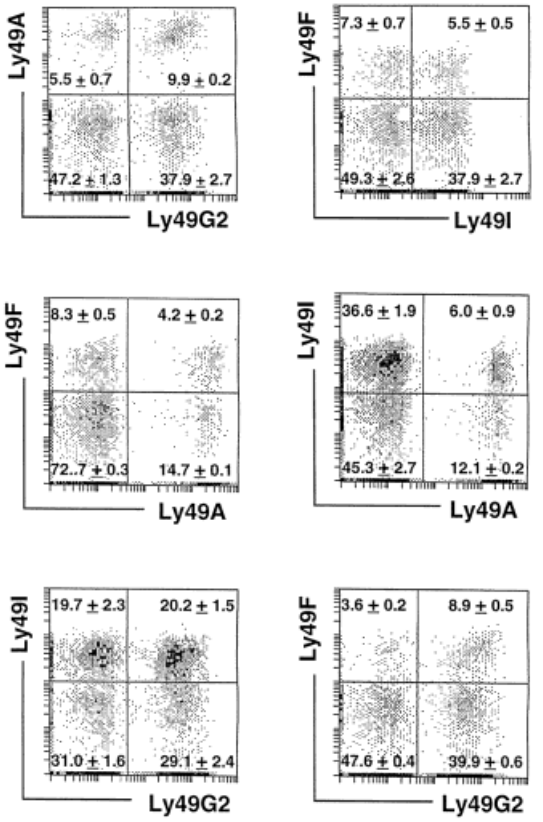

Fig. 1. Extensive co-expression of Ly49A, -G2, -I and -F on NK cells. Nylon-wool passed splenocytes from B10 mice were four-color-stained with mAb specific for NK1.1, CD3/CD8 and Ly49A (mAb JR9-318), Ly49G2 (mAb 4D11), Ly49I (mAb YLI), and/or Ly49F (mAb HBF), respectively. Representative dot plots for gated NK1.1$^+$ CD3$^-$CD8$^-$ NK cells are shown. Numeric values are means ± SEM of three individual mice.

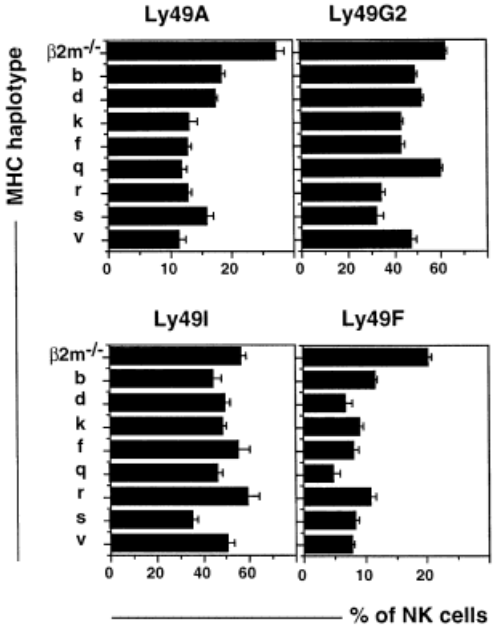

Fig. 2. The fraction of splenic NK cells bearing inhibitory Ly49 family members is influenced by MHC. Splenocytes of B6 β2m$^{-/-}$, B10 (b), B10.D2 (d), B10.BR (k), B10.M (f), B10.Q (q), B10.RIII (r), B10.S (s), or B10.SM (v) mice (MHC haplotype in parentheses) were stained as in Fig. 1. The frequencies of Ly49A$^+$, -G2$^+$, I$^+$ or -F$^+$ NK cells are shown as means ± SEM of three independent determinations.

analyses to more MHC haplotypes and more individual Ly49 receptors with defined MHC specificities.

In class I$^+$ mice, the frequency of NK cells expressing Ly49A, -F, -G2 or -I varied within a 2–3-fold range among the eight different haplotypes. Individual Ly49 receptors were affected differently by the MHC haplotypes of the hosts. For example, Ly49A expression was least frequent in H-2^v mice, whereas Ly49G2, -F and -I were expressed at intermediate frequencies in H-2^v mice. It is of interest to note that the representation of Ly49 receptors does not always inversely correlate with the order of binding to MHC alleles as determined by a semiquantitative adhesion assay [13]. For example, Ly49 binds more strongly to H-2^d cells than to any of the other MHC haplotypes tested, but is represented in H-2^d mice at a rather high frequency when all eight haplotypes are compared (see Discussion). Together, the data show that expression of four individual inhibitory Ly49 receptors is affected diversely by a broad range of MHC class I alleles.

2.2 MHC-independent and MHC-dependent deviations from the product rule

As shown in Fig. 1, co-expression of all combinations of Ly49A, -F, -G2 and -I occurred in class I$^+$ (H-2^b) mice, and the numbers roughly approximate those predicted for the product rule. For a more detailed assessment of receptor independence, we determined whether non-MHC factors influence Ly49 co-expression. Thus, using class I-deficient β2m$^{-/-}$ mice, we compared the fractions of NK cells expressing any pair of the four receptors to the product rule predictions (Fig. 3). The co-expression frequencies of Ly49F and Ly49I, and Ly49G2 and Ly49I precisely matched the product-rule predictions for independent expression. Co-expression of Ly49A and Ly49I was slightly lower than predicted, but this difference was not seen in a replicate experiment (data not shown). Interestingly, co-expression of Ly49A and Ly49G2, Ly49A and Ly49F, and Ly49F and Ly49G2 was more frequent than predicted by the product rule, resulting in an

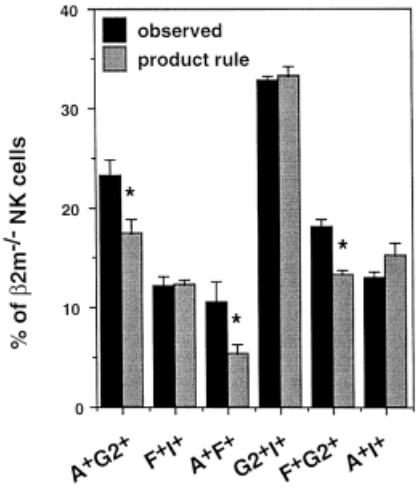

Fig. 3. MHC I-independent co-expression of inhibitory Ly49 receptor pairs is stochastic for some receptor combinations, and biased for others. Frequencies of NK cells co-expressing the indicated pairs of Ly49 receptors (determined as in Fig. 1, black bars) are compared to the expected frequencies of receptor combinations assuming independent distribution ("product rule", grey bars). Means ± SD of three animals are shown. * Statistically significant differences between observed values and product rule predictions, $p < 0.05$, as determined with Student's *t*-test. For the pair Ly49A-Ly49I, the difference was significant in this experiment, but not in a repeat experiment.

observed/expected ratio greater than one as summarized in Table 1. Similar deviations from the product rule were observed for these pairs in $K^{b-/-}D^{b-/-}$ B6 mice, which lack all expression of classical class I molecules (data not shown). Therefore, non-MHC factors influence the co-expression of some but not all Ly49 receptor combinations.

The non-MHC effects on the repertoire must be factored out when evaluating the effects of MHC expression on the NK repertoire. To do so, the observed to expected ratios for each receptor pair in NK cells from eight MHC congenic mouse strains were compared to the ratios for the same pairs in class I-deficient mice. These data are summarized in Table 1. The numbers in parentheses have been normalized for the results in class I-deficient mice, so that a value of less than one indicates inhibition of co-expression compared to MHC-deficient mice, while a value greater than one indicates enhanced co-expression. Interestingly, MHC-specific deviations from independent co-expression were observed for all receptor pairs and for many, but not all MHC haplotypes. Most of the MHC alleles inhibited co-expression of Ly49A with other Ly49 receptors. Prominent effects were observed in the $H\text{-}2^d$ haplotype, which encodes the strong D^d Ly49A ligand, but not in $H\text{-}2^b$, which lacks strong Ly49A ligands. Importantly, inhibition occurred in some instances where only one of the receptors in the pair binds detectably to MHC, for example the Ly49A/G2 pair in the $H\text{-}2^{k,f,s,v}$ haplotypes. This result is predicted by the regulated sequential model but not by the selection model of

Table 1. Frequencies of NK cells expressing combinations of two Ly49 receptors[a)]

MHC haplo.	$Ly49A^+G2^+$	$Ly49F^+I^+$	$Ly49A^+F^+$	$Ly49G2^+I^+$	$Ly49F^+G2^+$	$Ly49A^+I^+$
	Obs/Exp (Norm)	Obs/Exp (Norm)	Obs/Exp (Norm)	Obs/Exp (Norm)	Obs/Exp (Norm)	Obs/Exp (Norm)
$\beta2m^{-/-}$	1.33 ± 0.02	0.99 ± 0.06	1.95 ± 0.05	0.98 ± 0.02	1.37 ± 0.02	0.84 ± 0.04
b	1.30 ± 0.05 (0.98)	0.99 ± 0.05 (1.00)	1.79 ± 0.11 (0.92)$^+$	1.03 ± 0.01 (1.05)*	1.47 ± 0.05 (1.07)*	0.77 ± 0.03 (0.92)*
d	0.77 ± 0.02 (0.58)*	1.0 ± 0.12 (1.03)	1.11 ± 0.10 (0.57)*	0.98 ± 0.03 (1.00)	1.08 ± 0.08 (0.79)*	0.71 ± 0.02 (0.85)*
k	1.12 ± 0.01 (0.84)*	0.85 ± 0.04 (0.86)	1.54 ± 0.10 (0.79)*	0.97 ± 0.03 (0.99)	1.41 ± 0.08 (1.03)	0.67 ± 0.04 (0.80)*
f	0.97 ± 0.08 (0.72)*	1.13 ± 0.08 (1.14)	1.12 ± 0.06 (0.57)*	0.99 ± 0.04) (1.01)	1.23 ± 0.02 (0.90)*	0.70 ± 0.03 (0.83)*
q	1.21 ± 0.01 (0.91)	1.16 ± 0.07 (1.17)*	1.61 ± 0.48 (0.82)	0.95 ± 0.02 (0.97)	1.38 ± 0.16 (1.01)	0.62 ± 0.10 (0.73)*
r	1.28 ± 0.17 (0.96)	1.05 ± 0.05 (1.06)	1.26 ± 0.24 (0.65)*	1.04 ± 0.03 (1.06)*	1.41 ± 0.09 (1.03)	0.92 ± 0.02 (1.09)*
s	1.15 ± 0.01 (0.86)*	1.29 ± 0.09 (1.30)*	1.56 ± 0.16 (0.80)*	1.08 ± 0.02 (1.10)*	1.24 ± 0.05 (0.91)*	0.89 ± 0.04 (1.06)
v	0.94 ± 0.02 (0.71)*	1.05 ± 0.13 (1.06)	1.51 ± 0.21 (0.77)*	0.99 ± 0.02 (1.01)	1.27 ± 0.06 (0.93)*	0.55 ± 0.03 (0.65)*

a) Numbers indicate ratio ± SD of "observed *vs.* expected" frequencies. The numbers in parentheses have been normalized for the ratio in $\beta2m^{-/-}$ mice (mean of "observed *vs.* expected" from class I^+ mice/mean of "observed *vs.* expected" from $\beta2m^{-/-}$ mice). *Statistically significant difference compared to $\beta2m^{-/-}$ mice, $p < 0.05$, as determined with Student's *t*-test.

NK cell repertoire formation (see Discussion). However, some haplotypes with relatively strong Ly49A ligands had little effect on co-expression with Ly49A. For example, co-expression of Ly49A and Ly49I was not inhibited in H-2^r mice, where strong ligands for both receptors are present [13] (see Discussion).

In a few cases, there was a statistically significant enhancement in receptor co-expression due to MHC expression, but only one of these represented a relatively large effect. Co-expression of Ly49F and Ly49I was significantly elevated in H-2^s mice compared to class I-deficient mice. The results raise the possibility that that MHC can also positively influence the co-expression of Ly49 receptors in rare instances.

2.3 Evidence for the regulated sequential model from a transgenic mouse model

The data as a whole indicate that MHC inhibition of Ly49 receptor co-expression is common and fulfill a prediction of the regulated sequential model of repertoire formation, but the complexity of the system is such that we turned to another approach to more directly test the regulated sequential model. The selection model predicts selection against NK cells that co-express two (or more) receptors, but only if both of the receptors are self-MHC specific. The regulated sequential model predicts that expression of one self-specific receptor will inhibit subsequent expression of another receptor, whether the second receptor is self MHC-specific or not. Thus, the two models offer different predictions for the outcome of an experiment in which a self-MHC specific Ly49A transgene is expressed in all NK cells at an early stage of development. We previously characterized such a transgenic line, in which a Ly49A cDNA is expressed by all NK cells at physiological levels very early in NK cell differentiation [31]. The regulated sequential model predicts that the transgene will inhibit expression of both non-self MHC-specific and self-specific endogenous Ly 49 receptors, whereas the selection model predicts reduced usage of only self-specific receptors (see Fig. 4). Previous studies demonstrated that the transgene caused a reduced usage of endogenous Ly49G2 in H-2^d mice, but had little or no effect in H-2^b- or class I-deficient mice, in which no or only weak Ly49A ligands are expressed [28]. Ly49A binds quite well to both H-2^d and H-2^f, while Ly49G2, Ly49F and Ly49I bind to H-2^d and not H-2^f [13]. Ly49A-transgenic mice were crossed to a H-$2^{d/d}$ or H-$2^{f/f}$ B6/B10 background, and Ly49G2, -F and -I expression by splenic NK cells was determined. As predicted by both models, expression of the Ly49A transgene resulted in reduced usage of Ly49F, Ly49G2 and (to a lesser extent) Ly49I in H-2^d mice, which express ligands for all of these receptors (Fig. 5). Strikingly, a nearly iden-

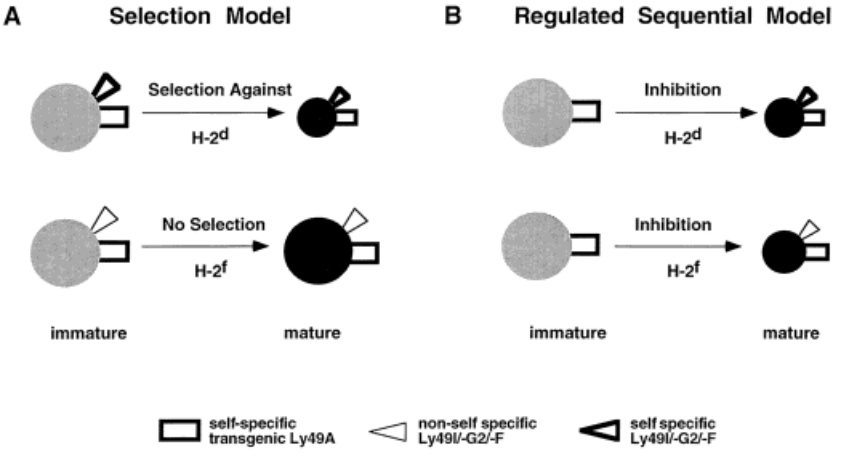

Fig. 4. The Ly49 repertoire in Ly49-A-transgenic H-2^d and H-2^f mice distinguishes the "selection" and "regulated sequential" models of Ly49 repertoire generation. According to the selection model (A), immature NK cells expressing transgenic Ly49A and an initially arbitrary set of self- or non-self MHC specific Ly49 receptors are subjected to selection against cells expressing "too many" self-specific receptors, as in the case of transgenic Ly49A and Ly49G2, -F or -I in H-2^d mice. In H-2^f mice, Ly49G2, -F or -I do not recognize self-MHC, and therefore, the generation of NK cells expressing transgenic Ly49A together with these receptors is neutral. (B) According to the "regulated sequential" model, early H-2^d or H-2^f recognition by transgenic Ly49A reduces the likelihood of subsequent expression of other inhibitory Ly49 receptors, irrespective of their MHC specificity. Therefore, expression of Ly49G2, -F or -I should be similarly infrequent in H-2^d and H-2^f mice. (A; B) Decreased cell size symbolizes contraction of the respective NK compartment due to MHC-specific education.

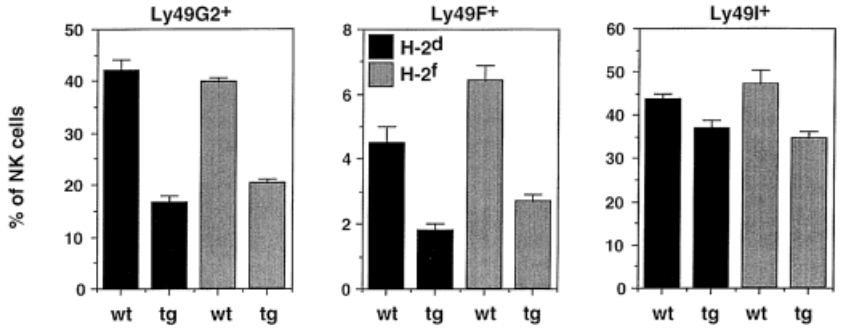

Fig. 5. Similar Ly49G2, -I and -F expression on NK cells from Ly49A transgenic H-2^{d} and H-2^{f} mice. Nylon-wool passed spleen cells from Ly49A-transgenic (tg) or non-transgenic (wt) H-2$^{d/d}$ (black bars) or H-2$^{f/f}$ mice (grey bars) were stained with mAb specific for Ly49G2 (4D11), Ly49F (HBF) or Ly49I (YLI). Mean results for gated NK1.1^{+} CD3^{-} cells ± SEM (n = 3) are shown.

tical effect of the Ly49A transgene was observed in H-2^{f} mice, despite the lack of ligands for Ly49G2, -F and -I in these mice. In contrast, the transgene had no effect on expression of Ly49G2 in class I-deficient mice [28], demonstrating that ligand-binding is necessary in order for the transgene-encoded receptor to exert its effects. These data strongly support the regulated sequential model of MHC specific NK repertoire generation.

3 Discussion

Here, we studied the co-expression frequencies of inhibitory Ly49 receptors on splenic NK cells from mice that differ solely in MHC expression. Compared to previous studies in this area [17, 24, 26] the present study has several notable features. First, we developed and employed the novel mAb YLI and HBF that specifically recognized Ly49I and Ly49F, respectively, to detect NK cell surface expression of these molecules. Second, since expression of Ly49 receptors by NKT cells differs in important respects from expression of these receptors by conventional NK cells, we used four-color flow-cytometry to gate NKT cells out of our analyses. Third, we employed a panel of eight MHC-congenic mouse strains and recently acquired knowledge of MHC-Ly49 specificity to comprehensively analyze the effect of MHC on receptor co-expression. Finally, we employed a well-studied Ly49A transgenic mouse model, in combination with the new mAb, to test a major prediction of the regulated sequential model of NK cell repertoire formation.

Extensive co-expression of inhibitiry Ly49 family members on the surface of NK cells has previously been shown for Ly49A, -G2 and -C/I [10, 11, 17, 26, 32]. It was noted that the frequency of NK cells co-expressing two receptors approximates the product rule, though deviations occur [15]. Previous studies have documented biases in co-expression of activating Ly49D and Ly49H as well as co-expression of Ly49H with Ly49C/I or -G2, but not Ly49A [33]. In the same study, and in accordance with previous findings [34], co-expression of the Qa-1 receptor CD94/NKG2 with Ly49A was found to be unbiased, while co-expression of CD94/NKG2 with Ly49G2 was somewhat disfavored. Since these studies did not include analysis of MHC class I-deficient or MHC-disparate mice, and the specificity of Ly49H is unknown, it was unclear whether the biases arose intrinsically or as a result of MHC interactions in the formation of the repertoire.

Interestingly, we found that in the absence of class I, co-expression of some receptor pairs closely matched the product rule predictions (Ly49F/Ly49I and Ly49G2/Ly49I), while co-expression of other pairs, such as Ly49A/Ly49F, Ly49A/Ly49G2 and Ly49F/Ly49G2, were all significantly higher than expected. The results are reminiscent of the recent finding that co-expression in F1 hybrid mice of two Ly49A alleles, or two Ly49G2 alleles, occur at a higher than expected frequency [22]. Therefore, the “intrinsic” factors that bias co-expression of different Ly49A alleles may also bias co-expression of Ly49A with other Ly49 genes.

It is attractive to speculate that the MHC-independent differences in receptor co-expression occur because of heterogeneity in precursor NK cells with respect to the likelihood of expression of different receptor genes. For example, a subset of precursor NK cells may express relatively high levels of nuclear factors, signaling molecules or other cellular components that are necessary for expression of Ly49A, Ly49F and Ly49G2. While the receptors would still be expressed in a variegated fashion in this subset, the higher availability of necessary factors could led to a high degree of co-expression of these

receptors in this subset compared to other NK cells. As a result, co-expressing cells would be more frequent among all NK cells than predicted by the product rule. One might speculate further that the expression of Ly49I is regulated by factors or signaling pathways that are at least partially distinct from those regulating the other three receptors, and which are randomly distributed in NK precursor cells. This would lead to a more or less random overlap between Ly49I and the other receptors. Only one nuclear factor has so far been implicated in Ly49 gene regulation, the transcription factor TCF-1 [35]. Deficiency of TCF-1 abrogated Ly49A expression, but resulted in unchanged or even increased percentages of Ly49G2$^+$ and Ly49C/I$^+$ NK cells. Therefore it is unlikely that restricted expression of TCF-1 in a subset of NK cell precursors could by itself account for the enhanced co-expression of Ly49A and Ly49G2. It remains possible that other factors necessary for expression of Ly49G2 (and Ly49F) are co-distributed with TCF-1 in NK cell precursors, or that TCF-1, while important, is not itself responsible for enhanced receptor co-expression.

Superimposed on intrinsic phenomena, MHC molecules clearly impact Ly49 co-expression (Table 1). In only one case, the Ly49F/I pair in H-2^s mice, did MHC expression substantially elevate receptor co-expression. At present, we do not have an explanation for this effect. One might speculate that H-2^s encodes a ligand for a stimulatory receptor that is expressed in a subset of NK cells and that induces expression of both of these receptors.

In most cases where an effect was observed, MHC interactions resulted in decreased receptor co-expression compared to class I-deficient mice. This outcome is in line with previous findings that receptor co-expression is limited by interactions with MHC class I molecules. One of the most notable features of the data, however, is that MHC haplotypes which did not detectably bind certain Ly49 receptors nevertheless impacted co-expression of these receptors with other Ly49 molecules. For example, Ly49F does not bind detectably to H-2^f or H-2^r, but co-expression of Ly49A and Ly49F was strongly inhibited in both H-2^f and H-2^r mice. These data are difficult to reconcile with the selection model of Ly49 receptor co-expression, in which the summed reactivity of two receptors is the determinant of whether cells will be selected against. In contrast, these data are consistent with the sequential regulated model, in which the reactivity with MHC of receptors expressed early in the sequence determines whether other receptors will be subsequently expressed, regardless of whether the latter receptors bind MHC.

We previously reported evidence that Ly49A is expressed relatively early in NK cell development [23, 24] (though another group obtained different results [25]). Therefore one might predict that the reactivity of this receptor with MHC would have a significant impact on co-expression of other subsequently expressed receptors. The "avidity" of different Ly49 receptors for each of the eight MHC haplotypes studied was previously determined in a cell-cell binding assay, where it was shown that Ly49A interacts with the eight haplotypes in the order d > r > (f) > k > v > q > s > b [13]. There appeared to be some relationship between MHC avidity and the impact of the MHC haplotype on receptor co-expression, but the relationship was inexact and did not hold for all receptor pairs. For example, the three MHC haplotypes in which co-expression of Ly49A/F was most affected were those haplotypes that bind most tightly to Ly49A (H-2$^{d,\ f\ and\ r}$). In the case of the Ly49A/G pair, two of the three MHC haplotypes showing a strong impact, H-2^d and H-2^f, were among the strongest binders to Ly49A. On the other hand, H-2^r, the second strongest binder to Ly49A, had no impact on the co-expression of Ly49A/G2, and the Ly49A/I pair showed no discernable correlation with MHC binding to either Ly49A or Ly49I. We suspect that these discrepancies arise because the overall outcome is determined by the complex interplay of all the receptors, including those not studied here, and depends on the order of expression of each receptor and its reactivity with MHC. Because such an analysis would be complex even if all the relevant data were available, we used a more direct transgenic approach to test a major prediction of the regulated sequential model.

The transgenic studies investigated the effects of a Ly49A transgene in H-2^f mice. Ly49A binding to H-2^f was shown with a cell-cell adhesion assay as well as a functional assay in which H-2^f stimulator cells inhibited Ly49A-transgenic T cells as compared to non-transgenic T cells [13, 29]. On the other hand, recombinant biotinylated Ly49A failed to bind to H-2^f [36], possibly reflecting a lower avidity of recombinant Ly49A to H-2^f as compared to the membrane-bound transgene. Significantly, we observed a substantial effect of the Ly49A transgene on expression of endogenous Ly49G2, -F and -I by splenic NK cells from H-2^f mice (Fig. 5). Since the transgene does not affect Ly49G2 expression in class I-deficient mice [28], the data indicate that the Ly49A transgene recognizes H-2^f class I molecules in a developmentally relevant context.

In contrast to Ly49A, no binding of Ly49G2, -F and -I to H-2^f was observed in the cell-cell adhesion assay [13]. Nevertheless, Ly49A transgene expression resulted in reduced co-expression of each of these receptors in H-2^f mice, of a similar magnitude as observed in H-2^d mice, which express strong ligands for both receptors. These data fulfill a central prediction of the regulated sequential

model of Ly49 repertoire generation, since this model anticipates that engagement of the transgene-encoded receptor will inhibit subsequent expression of other receptors regardless of their reactivity with self-MHC molecules. The selection model, in contrast, predicts that NK cells with a given receptor pair will be selected against only if both receptors react with self-MHC molecules. As this report was prepared for publication, Fahlen et al. reported that a Ly49C transgene results in reduced usage of Ly49G2 in mice expressing H-2^b (which binds to Ly49C), but not in class I-deficient mice [37]. Our data significantly extend these results by demonstrating a similar effect with a different transgene, and by showing that three different Ly49 receptors were affected by the transgene.

A notable feature of the transgenic data was that Ly49A transgene expression inhibited Ly49I expression to a substantially smaller extent than it inhibited Ly49G2 or -F expression. This was true even in mice expressing H-2^d, which binds significantly to both Ly49A and -I. The data suggest that Ly49 genes differ in their sensitivity to regulation by prior receptor engagement. Perhaps the Ly49I gene is less dependent than the other genes on factors that are down-regulated as a result of prior receptor engagement, and is therefore less affected. As mentioned above, evidence suggests that different Ly49 genes are regulated by different factors, since TCF-1 deficiency prevented expression of Ly49A but not several other receptors [35]. The lower responsiveness of Ly49I to regulation may also help explain why receptor pairs including Ly49I were generally (but not always) less affected than other pairs in various MHC-congenic mice (Table 1).

In conclusion, we have provided the most comprehensive characterization to data of the Ly49 receptor repertoire in MHC-different mice. The data indicate that MHC interactions often inhibit receptor co-expression, and provide evidence consistent with the regulated sequential model of NK cell repertoire formation. More direct evidence for the regulated sequential model came from the transgenic studies. In further studies, it will be interesting to unravel the molecular mechanisms that underlie the regulation of receptor expression in this system.

4 Materials and methods

4.1 Mice

C57BL/6J (B6) mice were bred at the animal facilities of the University of California, Berkeley. C57BL/10J (H-2^b, B10), B10.D2/nSnJ (H-2^d, B10.D2), B10.BR/SgSnJ (H-2^k, B10.BR), B10.M/Sn (H-2^f, B10.M), B10.Q/SgJ (H-2^{q_1}, B10.Q), B10.RIII (71NS)/Sn (H-2^r, B10.RIII) B10.S/SgMcdJ (H-2^s, B10.S) and B10.SM (70NS)/Sn (H-2^v, B10.SM) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). B6 and B10 mice express identical NK cell receptors [38]. The generation of $\beta 2m^{-/-}$ mice and Ly49A-transgenic mice (line 2) on the B6 background has been described previously [17, 28]. Ly49A-transgenic B6 mice were crossed to B10.M (H-2^f) mice to generate Ly49A-transgenic B6/B10 H-$2^{b/f}$ F1 mice which were subsequently crossed to B10.M mice to generate Ly49A-transgenic H-$2^{f/f}$ mice. For analysis of the Ly49 repertoire, 8–12-week-old mice were used throughout the study.

4.2 Antibodies and staining reagents

Anti-Ly49 mAb used in this study were: HBF (anti-Ly49F), YLI (anti-Ly49I) [39], 4D11 (anti-Ly49G2, ATCC, Manassas, WI), JR9-318 (anti-Ly49A, kindly provided by J. Roland). Antibodies were purified from cell culture supernatants on protein A- or protein G-Sepharose columns and conjugated to biotin or FITC according to standard methods. Anti-NK1.1-PE was from Pharmingen (San Diego, CA), anti-CD3-Tricolor (TC) and anti-CD8-TC from Caltag (San Francisco, CA). Biotin conjugates were developed with SA-613 (Gibco BRL, Gaithersburg, MD) for four-color analysis or with SA-TC (Caltag) for three-color analysis.

4.3 Stainings and flow cytometry

Spleen cell suspensions were depleted of red blood cells by osmotic shock and passaged over nylon-wool columns to enrich for NK cells. All staining reactions were performed in the dark on ice in PBS/3 % FCS/0.02 % sodium azide with saturating amounts of reagents. For four-color analysis, 1×10^6 cells were stained with biotin-conjugated anti-Ly49A, -F, -G2 or -I followed by extensive washing and anti-NK1.1-PE, anti-CD3/8-TC, anti-Ly49A-FITC, anti-Ly49F-FITC or anti-Ly49G2-FITC, respectively, and SA-613. Samples were analyzed on an EPICS XL flow cytometer (Coulter, Hialeah, FL) with a gate on live cells based on light scatter profile. WinMDI® software was used for data display. All results are shown as $\log_{10}$ fluorescence intensities on a four-decade scale.

Acknowledgements: We thank Ann Lazar and Scot Liu for expert technical assistance. This work was supported by DFG grant HA 2456/3-1 to T. H. and NIH grants to D. H. R.

References

1 **Hoglund, P., Sundbäck, J., Olsson-Alheim, M. Y., Johansson, M., Salcedo, M., Öhlén, C., Ljunggren, H. G., Sentman, C. and Karre, K.,** Host MHC class I gene control of NK cell specificity in the mouse. *Immunol. Rev.* 1997. **155:** 11–28.

2 **Raulet, D. H., Vance, R. E. and McMahon, C. W.** Regulation of the natural killer cell receptor repertoire. *Annu. Rev. Immunol. 2001.* **19:** 291–330.

3 **Tomasello, E., Blery, M., Vely, E. and Vivier, E.,** Signaling pathways engaged by NK cell receptors: double concerto for activating receptors, inhibitory receptors and NK cells. *Semin. Immunol.* 2000. **12:** 139–147.

4 **Lanier, L. L.,** On guard-activating NK cell receptors. *Nature Immunol.* 2001. **2:** 23–27.

5 **Ljunggren, H. G. and Karre, K.** In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol. Today* 1990. **11:** 237–244.

6 **Lopez-Botet, M., Llano, M., Navarro, F. and Bellon, T.** NK cell recognition of non-classical HLA class I molecules. *Semin. Immunol.* 2000. **12:** 109–119.

7 **Takei, F., Brennan, J. and Mager, D. L.,** The Ly49 family: genes proteins and recognition of class I MHC. *Immunol. Rev.* 1997. **155:** 67–77.

8 **McQueen, K. L., Freeman, J. D., Takei, F. and Mager, D. L.,** Localization of five new Ly49 genes, including three closely related to Ly49c. *Immunogenetics* 1998. **48:** 174–183.

9 **Karlhofer, F. M., Ribaudo, R. K. and Yokoyama, W. M.,** MHC class I alloantigen specificity of Ly-49$^+$ IL-2 activated natural killer cells. *Nature* 1992. **358:** 66–70.

10 **Yu, Y. Y., George, T., Dorfman, J., Roland, J., Kumar, V. and Bennett, M.,** The role of Ly49A and 5E6 (Ly49C) molecules in hybrid resistance mediated by murine natural killer cells against normal T cell blasts. *Immunity* 1996. **4:** 67–76.

11 **Mason, L. H., Ortaldo, J. R., Young, H. A., Kumar, V., Bennett, M. and Anderson, S. K.,** Cloning and functional characteristics of murine LGL-1: a member of the Ly-49 gene family (Ly-49G2). *J. Exp. Med.* 1995. **182:** 293–303.

12 **Yokoyama, W. M.,** Natural killer cell receptors specific for major histocompatibility complex class I molecules. *Proc. Natl. Acad. Sci. USA* 1995. **92:** 3081–3085.

13 **Hanke, T., Takizawa, H., McMahon, C. W., Busch, D. H., Pamer, E. G., Miller, J. D., Altman, J. D., Liu, Y., Cado, D., Lemonnier, F., Bjorkman, P. J. and Raulet, D. H.,** Direct assessment of MHC class I binding by seven Ly49 inhibitory NK cell receptors. *Immunity* 1999. **11:** 67–77.

14 **Johansson, M. H., Hoglund, E., Nakamura, M. C., Ryan, J. C. and Hoglund, P.,** $\alpha1/\alpha2$ domains of H-2D^d but not L^d, induce "missing self" reactivity *in vivo* – No effect of H-2L^d on protection against NK cells expressing the inhibitory receptor Ly49G2. *Eur. J. Immunol.* 1998. **28:** 4198–4206.

15 **Raulet, D. H., Held, W., Correa, I., Dorfman, J., Wu, M.-F. and Corral, L.,** Specificity, tolerance and developmental regulation of natural killer cells defined by expression of class I-specific Ly49 receptors. *Immunol. Rev.* 1997. **155:** 41–52.

16 **Uhrberg, M., Valiente, N., Shum, B., Shilling, H., Lienert-Weidenbach, K., Corliss, B., Tyan, D., Lanier, L. and Parham, P.,** Human diversity in killer cell inhibitory receptor genes. *Immunity* 1997. **7:** 753–763.

17 **Held, W., Dorfman, J. R., Wu, M.-F. and Raulet, D. H.,** Major histocompatibility complex class I-dependent skewing of the natural killer cell Ly49 receptor repertoire. *Eur. J. Immunol.* 1996. **26:** 2286–2292.

18 **Kubota, A., Kubota, S., Lohwasser, S., Mager, D. L. and Takei, D.,** Diversity of NK cell receptor repertoire in adult and neonatal mice. *J. Immunol.* 1999. **163:** 212–216.

19 **Held, W., Roland, J. and Raulet, D. H.,** Allelic exclusion of Ly49 family genes encoding class I-MHC-specific receptors on NK cells. *Nature* 1995. **376:** 355–358.

20 **Held, W. and Raulet, D. H.,** Expression of the *Ly49A* gene in murine natural killer cell clones is predominantly but not exlusively mono-allelic. *Eur. J. Immunol.* 1997. **27:** 2876–2884.

21 **Held, W. and Kunz, B.,** An allelic-specific, stochastic gene expression process controls the expression of multiple Ly49 family genes and generates a diverse, MHC-specific NK cell receptor repertoire. *Eur. J. Immunol.* 1998. **28:** 2407–2416.

22 **Tanamachi, D. M., Hanke, T., Takizawa, H., Jamieson, A. M. and Raulet, D. H.,** Expression of Natural Killer Receptor Alleles at Different Ly49 Loci Occurs Independently and Is Regulated by Major Histocompatibility Complex Class I Molecules. *J. Exp. Med.* 2001. **193:** 307–316.

23 **Dorfman, J. R. and Raulet, D. H.,** Acquisition of Ly49 receptor expression by developing natural killer cells. *J. Exp. Med.* 1998. **187:** 609–618.

24 **Roth, C., Carlyle, J. R., Takizawa, H. and Raulet, D. H.,** Clonal acquisition of inhibitory Ly49 receptor on developing NK cells is successively restricted and regulated by stromal class I MHC. *Immunity* 2000. **13:** 143–153.

25 **Williams, N. S., Kubota, A., Bennett, M., Kumar, V. and Takei, F.** Clonal analysis of NK cell development from bone marrow progenitors *in vitro:* orderly acquisition of receptor gene expression. *Eur. J. Immunol.* 2000. **30:** 2074–2082.

26 **Salcedo, M., Diehl, A. D., Olsson-Alheim, M. Y., Sundbäck, J., Van Kaer, L., Karre, K. and Ljunggren, H.-G.,** Altered expression of Ly49 inhibitory receptors on natural killer cells from MHC class I deficient mice. *J. Immunol.* 1997. **158:** 3174–3180.

27 **Dorfman, J. R., Zerrahn, J., Coles, M. C. and Raulet, D. H.,** The basis for self-tolerance of natural killer cells in $\beta2m^-$ and TAP-1$^-$ mice. *J. Immunol.* 1997. **159:** 5219–5225.

28 **Held, W. and Raulet, D. H.,** Ly49A transgenic mice provide evidence for a major histocompatibility complex-dependent education process in NK cell development. *J. Exp. Med.* 1997. **185:** 2079–2088.

29 **Hanke, T. and Raulet, D. H.,** Cumulative inhibition of NK cells and T cells resulting from engagement of multiple inhibitory Ly49 receptors. *J. Immunol.* 2001. **166:** 3002–3007.

30 **Vance, R. E. and Raulet, D. H.,** Toward a quantitative analysis of the repertoire of class I MHC-specific inhibitory receptors on natural killer cells. *Curr. Top. Microbiol. Immunol.* 1998. **230:** 135–160.

31 **Held, W., Cado, D. and Raulet, D. H.,** Transgenic expression of the Ly49A natural killer cell receptor confers class I major histocompatibility complex (MHC)-specific inhibition and prevents bone marrow allograft rejection. *J. Exp. Med.* 1996. **184:** 2037–2041.

32 **Brennan, J., Mager, D., Jefferies, W. and Takei, F.,** Expression of different members of the Ly-49 gene family defines distinct natural killer cell subsets and cell adhesion properties. *J. Exp. Med.* 1994. **180:** 2287–2295.

33 **Smith, H. R., Chuang, H. H., Wang, L. L., Salcedo, M., Heusel, J. W. and Yokoyama, W. M.,** Nonstochastic coexpression of activation receptors on murine natural killer cells. *J. Exp. Med.* 2000. **191:** 1341–1354.

34 **Vance, R. E., Kraft, J. R., Altman, J. D., Jensen, P. E. and Raulet, D. H.,** Mouse CD94/NKG2A is a natural killer cell receptor for the nonclassical MHC class I molecule Qa-1^b. *J. Exp. Med.* 1998. **188:** 1841–1848.

35 **Held, W., Kunz, B., Lowin-Kropf, B., van de Wetering, M. and Clevers, H.,** Clonal acquisition of the Ly49A NK cell receptor is dependent on the trans-acting factor TCF-1. *Immunity* 1999. **11:** 433–442.

36 **Chung, D. H., Natarajan, K., Boyd, L. F., Tormo, J., Mariuzza, R. A., Yokoyama, W. M. and Margulies, D. H.,** Mapping the ligand of the NK inhibitory receptor Ly49A on living cells. *J. Immunol.* 2000. **165:** 6922–6932.

37 **Fahlen, L., Lendahl, U. and Sentman, C. L.,** MHC class I-Ly49 interactions shape the Ly49 repertoire on murine NK cells. *J. Immunol.* 2001. **166:** 6585–6592.

38 **Yokoyama, W. M. and Seaman, W. E.,** The Ly-49 and NKR-P1 gene families encoding lectin-like receptors on natural killer cells: the NK gene complex. *Annu. Rev. Immunol.* 1993. **11:** 613–635.

39 **Coles, M C., McMahon, C. W., Takizawa, H. and Raulet, D. H.,** Memory CD8 T lymphocytes express inhibitory MHC-specific Ly49 receptors. *Eur. J. Immunol.* 2000. **30:** 236–244.

Correspondence: David H. Raulet, Department of Molecular and Cell Biology and Cancer Research Laboratory, University of California, Berkeley, CA 94720, USA
Fax: +1-510-642-1443
e-mail: raulet@uclink4.berkeley.edu