Natural killer cell education in mice with single or multiple major histocompatibility complex class I molecules

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The ability of murine NK cells to reject cells lacking self MHC class I expression results from an in vivo education process. To study the impact of individual MHC class I alleles on this process, we generated mice expressing single MHC class I alleles (K^b, D^b, D^d, or L^d) or combinations of two or more alleles. All single MHC class I mice rejected MHC class I–deficient cells in an NK cell–dependent way. Expression of K^b or D^d conveyed strong rejection of MHC class I–deficient cells, whereas the expression of D^b or L^d resulted in weaker responses. The educating impact of weak ligands (D^b and L^d) was further attenuated by the introduction of additional MHC class I alleles, whereas strong ligands (K^b and D^d) maintained their educating impact under such conditions. An analysis of activating and inhibitory receptors in single MHC class I mice suggested that the educating impact of a given MHC class I molecule was controlled both by the number of NK cells affected and by the strength of each MHC class I–Ly49 receptor interaction, indicating that NK cell education may be regulated by a combination of qualitative and quantitative events.

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Abbreviations used: CFSE, 5,6-carboxyfluorescein diacetate succinimidyl ester; DRI, down-regulating index; FI, fluorescence intensity.

The missing self hypothesis states that when NK cells fail to recognize endogenous MHC class I on cells, killing occurs (1). In contrast, when target cells express self MHC class I molecules, NK cells are inhibited. The missing self reaction is thought to regulate NK activity against virally infected or malignant cells, which frequently have down-modulated MHC class I expression to avoid T cell attack. In mice, the known inhibitory receptors that recognize MHC class Ia belong to the Ly49 family of lectin-like receptors (2). Humans lack functional Ly49 receptors and instead express the structurally unrelated but functionally analogous KIR family of inhibitory receptors (3). In addition, both mice and humans express the CD94/NKG2 heterodimer family that recognizes nonclassical MHC class Ib proteins carrying leader peptides derived from some MHC class Ia molecules (4). Inhibitory KIR and Ly49 receptors share structurally related immunoreceptor tyrosine–based inhibitory motif signaling motifs in their cytoplasmic domains, and their downstream signaling pathways are similar, including recruitment of Shp-1 leading to early dephosphorylation of activating signaling pathways (5).

Different NK cells express individual combinations of one to several Ly49 receptors, each of which can interact with several MHC class I molecules (6). This variable expression results in subsets of NK cells with different MHC class I specificities (7–9). To ensure proper missing self surveillance in vivo, it has been suggested that the NK cell system must be capable of recognizing loss of any self MHC class I molecule in vivo (10–12). Experiments with MHC class I transgenic mice have supported the existence of an education process controlling this reaction, because novel NK cell specificities arise upon introduction of a novel MHC class I molecule. When a D^d transgene was introduced into B6 mice (K^bD^b), NK cells emerged that specifically sensed the absence of D^d (13, 14). However, there are also data from transgenic systems showing that introduction of novel MHC class I transgenes may not always leave a strong imprint on the NK cell system, suggesting that

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different MHC class I molecules may not be equally important in NK cell education (15).

The MHC locus is polygenic and contains codominantly expressed alleles. Together with the large number of inhibitory Ly49 or KIR receptors, this multiplicity creates a significant complexity, making attempts to delineate the roles of individual receptor–ligand interactions in NK cell education difficult. However, this question is of importance, because viral infections and malignant transformation often downmodulate individual MHC class I alleles to varying degrees (16–18). This selectivity could serve to avoid MHC class I–restricted T cell responses but may also affect the strength of NK cell rejection of the infected target.

In the present study, we have taken a genetic approach to investigate the relative roles of individual MHC class I molecules in NK cell education. To do so, we generated mouse strains expressing single MHC class I molecules and defined combinations of up to three MHC class I molecules (19, 20). We also used a recently developed technique for quantitatively measuring NK cell rejection in vivo based on fluorescent labeling of injected cells (21). Using these tools, we asked the following questions: (a) Would the four MHC class I molecules tested here be equally efficient in educating a missing self response when expressed separately? (b) Would the introduction of other MHC class I molecules influence the strength of that response? (c) Could the educating impact of a given MHC class I molecule be predicted from the effects of this MHC class I molecule on the expression of activating and inhibitory receptors? Our results demonstrate that individual MHC class I molecules exert a complex and dynamic control of NK cell education that depends on other coexpressed MHC class I molecules and involves a combination of qualitative and quantitative effects on Ly49 receptor expression.

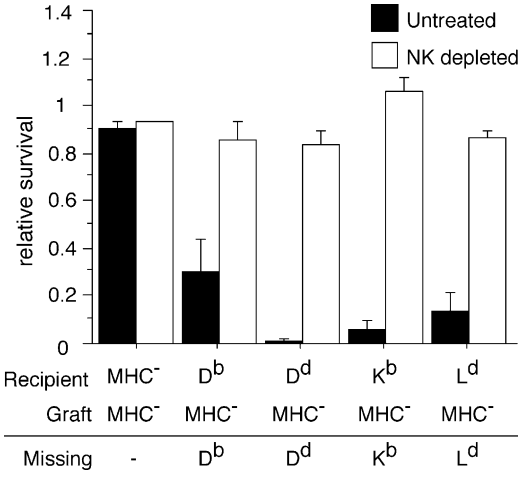

Figure 1. All single MHC class I mice reject MHC class I$^-$ grafts. MHC$^-$ spleen cells were mixed with spleen cells syngeneic to the recipient and coinjected i.v. into tilorone-treated recipients expressing either D^b, D^d, K^b, or L^d alone. Control and test cells were labeled with different doses of CFSE, allowing their subsequent distinction using FACS analysis. Eighteen hours later, the fraction of surviving MHC$^-$ cells in spleen was determined in each host by comparing the ratio between CFSE^{high} and CFSE^{low} cells in the recipient spleen. White bars show recipients in which NK cells were depleted before the transfer. All groups contained four mice except the NK-depleted MHC$^-$ and NK-depleted D^d groups, which contained two mice each. The relative survival of MHC$^-$ cells in D^b, D^d, K^b, and L^d mice was in all cases statistically different from that in MHC$^-$ mice ($P < 0.01$ for D^b; $P < 0.0001$ for D^d, K^b, and L^d). The survival was also significantly lower in D^d, K^b, and L^d mice compared with D^b mice and in K^b and D^d mice compared with L^d mice ($P < 0.05$). Results show averages of two experiments. Error bars show SDs. Graft, MHC class I phenotype on the test graft; missing, MHC lacking in this test graft in relation to the syngeneic control cells that were cotransferred.

RESULTS

Individual MHC class I molecules differ in their educating impact on NK cells

To study the educating impact of individual MHC class I alleles on NK cells, we generated mice expressing different single MHC class I molecules (Table I). Missing self rejection was tested in those mice using an in vivo assay based on labeling of the donor cells using 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) dye. In this assay, an individual internal control for each donor/host combination allows a quantitative measurement of rejection using a limited number of mice (21). Mice expressing K^b, D^b, L^d, or D^d as their only MHC class I molecule rejected grafted MHC class I–deficient cells in an NK cell–dependent fashion (Fig. 1), showing that they were independently able to educate a missing self response. However, the strength of rejection in the four recipients showed consistent differences. Although D^d and K^b conveyed strong missing self rejection, the educating impact of L^d and in particular D^b was weaker, resulting in less efficient rejection of MHC class I deficient cells (Fig. 1).

The educating impact of K^b is stronger than that of D^b when they are coexpressed

To test whether two MHC class I molecules with different educating impacts in single MHC class I mice would also

Table I. Mice used in this paper

Designation in this paper	Genotype
MHC$^-$	B6.$K^{b-/-}D^{b-/-}$
K^b	B6.$D^{b-/-}$
D^b	B6.$K^{b-/-}$
L^d	B6.$K^{b-/-}D^{b-/-}L^{d+}$
D^d	B6.$K^{b-/-}D^{b-/-}D^{d+}$
K^bD^b	B6
K^bL^d	B6.$D^{b-/-}L^{d+}$
K^bD^d	B6.$D^{b-/-}D^{d+}$
D^bL^d	B6.$K^{b-/-}L^{d+}$
D^bD^d	B6.$K^{b-/-}D^{d+}$
$K^bD^bL^d$	B6.L^{d+}
$K^bD^bD^d$	B6.D^{d+}

The designations used in this paper denote the MHC class Ia alleles expressed by the mouse strains. The genotypes of these mouse strains are also shown. All mice have the same genetic background (B6).

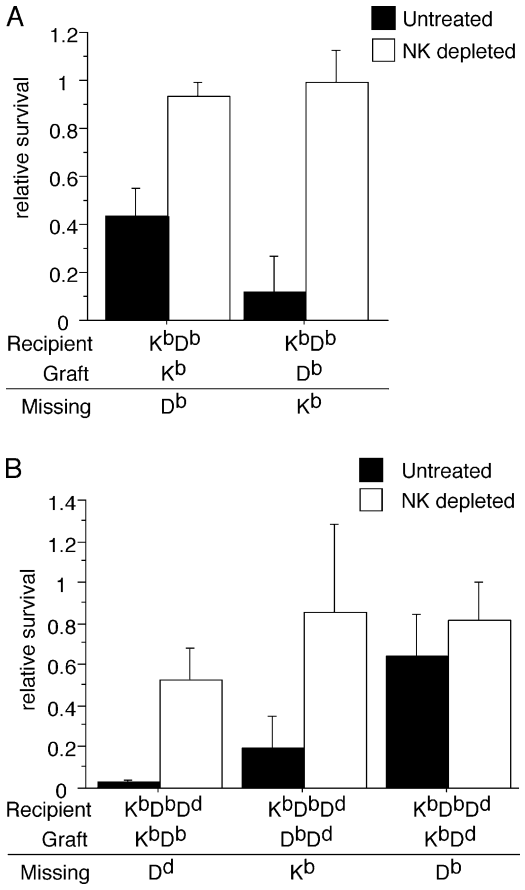

Figure 2. Influence on the educating impact by coexpressed MHC class I molecules. Syngeneic cells (K^bD^b) together with cells expressing either D^b or K^b were injected into K^bD^b mice as in Fig. 1. (A) K^b-negative grafts were more efficiently rejected than D^b-negative grafts in K^bD^b (B6) mice. The relative survival of target cells missing only K^b was significantly lower than the survival of cells missing D^b ($P < 0.001$). Results show averages of two experiments with four mice per group. Error bars show the standard deviations. (B) The lack of either K^b or D^d, but not D^b, on the grafted cells mediated strong rejection in $K^bD^bD^d$ (D8) mice. The relative survival of target cells missing D^b was significantly higher than the survival of cells missing D^d or K^b ($P < 0.05$). Results show averages of two experiments with three or four mice per group. Error bars show standard deviations.

have different educating impacts when they were coexpressed in one individual, we grafted cells lacking either D^b (weak educating impact) or K^b (strong educating impact) to K^bD^b (B6) recipients. Rejection of grafts lacking K^b in K^bD^b recipients was stronger than rejection of D^b-deficient grafts, suggesting that K^b had a stronger educating impact than D^b when the two molecules were coexpressed (Fig. 2 A).

K^b and D^d, but not D^b, educate strong rejection responses in $K^bD^bD^d$ mice

To investigate whether two MHC class I molecules displaying a strong educating impact when expressed alone would both remain strong if coexpressed, we used the MHC class I transgenic mouse strain D8 ($K^bD^bD^d$). Earlier transplantation studies using this strain have shown that the isolated lack of D^d on a graft is sufficient to trigger rejection of the grafted cells (13–15, 22). We asked whether K^b would also convey strong missing self activity in $K^bD^bD^d$ mice or whether the introduction of D^d reduced the importance of K^b. Our results showed that the absence of either K^b or D^d generated a strong rejection response in $K^bD^bD^d$ mice (Fig. 2 B). We conclude from this experiment that the NK cell system can be educated by several strong MHC ligands present in the host at the same time and can exhibit rejection responses of similar strengths against cells lacking each of them. Interestingly, injection of cells lacking D^b (but expressing K^b and D^d) evoked a very weak rejection response in $K^bD^bD^d$ recipients that did not reach statistical significance compared with the rejection in NK1.1-treated mice (Fig. 2 B). Thus, introduction of D^d on the B6 background reduced the educating impact of one allele (D^b) but left the other allele (K^b) unaffected.

The educating impacts of D^b and L^d are both attenuated in $K^bD^bL^d$ mice

In parallel with D^b, L^d also displayed a relatively weak educating impact in the single MHC class I mice as compared with K^b and D^d (Fig. 1). We therefore investigated whether L^d also would lose educating impact when coexpressed with other MHC class I molecules, a result that would explain the discrepancy between rejection of missing L^d in L^d single mice (Fig. 1), and results of previous experiments failing to reveal any rejection response against K^bD^b cells in $K^bD^bL^d$ mice (15). Our results in this study confirmed that missing L^d evoked a very weak rejection response in $K^bD^bL^d$ mice, demonstrating that L^d lost educating impact when coexpressed with K^b and D^b (Fig. 3). Interestingly, D^b also had lower educating impact in $K^bD^bL^d$ mice than in K^bD^b mice, revealing a second case in which the importance of D^b was diminished in the presence of two additional MHC class I molecules.

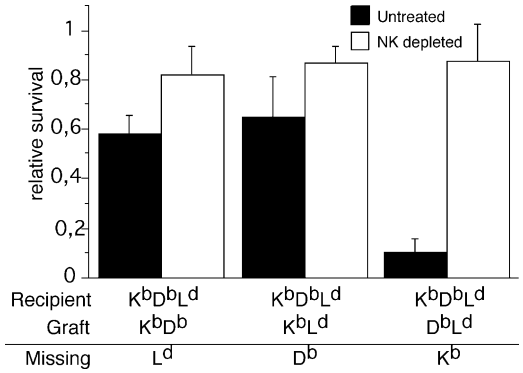

Figure 3. Missing K^b on the graft induces strong rejection in $K^bD^bL^d$ mice, whereas lack of either D^b or L^d results in a weaker response. Averages of two or three experiments with a total of four to six mice per group are shown. The survival of target cells missing K^b was significantly lower than the survival of cells missing L^d ($P < 0.05$) or D^b ($P < 0.0001$). Error bars show SDs.

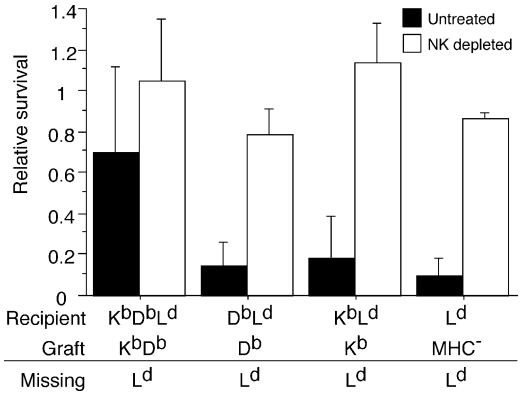

Figure 4. The lack of L^d evokes rejection of different strengths depending on the MHC background. Syngeneic cells and cells differing from the recipient only with regard to lack of L^d were coinjected into $K^bD^bL^d$, D^bL^d, K^bL^d, or L^d mice. The survival of cells missing only L^d was significantly better in $K^bD^bL^d$ mice than in D^bL^d or K^bL^d mice ($P < 0.01$). The bars show averages of two to four experiments with a total of three to seven mice in each group. The single L^d group includes also data from Fig. 1. Error bars show SDs.

Attenuation of the educating impact of L^d in $K^bD^bL^d$ mice requires coexpression of both K^b and D^b

To identify the MHC class I allele that was responsible for the loss of educating impact of L^d in $K^bD^bL^d$ mice, we grafted cells selectively lacking L^d to mice expressing L^d alone, L^d together with either K^b or D^b, or L^d in combination with both K^b and D^b. Our data showed that cells lacking L^d were strongly rejected in mice expressing L^d only and in mice expressing L^d in combination with either K^b or D^b (Fig. 4). In contrast, mice coexpressing L^d with both K^b and D^b were poor rejectors of L^d-deficient grafts (Fig. 4). Hence, only the combination of K^b and D^b diminished the response to missing L^d, whereas coexpression of only one of the two alleles still allowed missing L^d recognition in vivo.

The educating impact of a single MHC class I molecule cannot be predicted from the number of mature NK cells or from the expression pattern of activating receptors or the receptor KLRG1

To test whether the educating impact of a single MHC class I molecule was determined by the number of mature NK cells or by the expression of activating receptors on NK cells, we measured the number of NK cells, the frequencies and expression levels of the maturation marker CD11b (Mac-1) and of the activating receptors NK1.1, 2B4, CD16, NKG2D, and Ly49D on NK cells from mice in all four single MHC class I mice. No major differences in any of those parameters were found among the single MHC class I mice (Table II). We also failed to demonstrate a correlation between the educating impact and the frequency of NK cells that produced IFN-γ after stimulation by antibodies against NK1.1 (unpublished data), suggesting that mice carrying an MHC class I molecule of low educating impact did not contain a larger fraction of NK cells displaying global anergy to stimulation. The receptor KLRG1 is expressed in larger numbers and at higher levels on NK cells from B6 mice than on NK cells from MHC class I–deficient mice, and this receptor therefore has been suggested as a marker for NK cells active in missing self rejection (23). KLRG1 was up-regulated in all single MHC class I mice compared with MHC-deficient control mice (Table II). We did not find any differences in frequencies or cell surface expression of KLRG1 among the single MHC class I mice, arguing against the idea that differences in the number of educated NK cells determines educating impact.

Each single MHC class I mouse shows a unique expression pattern of MHC class I–specific inhibitory receptors

The cell surface levels of inhibitory Ly49 receptors are modulated in the presence of their MHC class I ligands, and a change in detectable Ly49 receptor levels at the cell surface compared with MHC^- mice (termed down-regulation in

Table II. Expression of different receptors on NK cells from single MHC class I mice

		Receptors						
	Mouse	NK1.1	CD11b	NKG2D	CD16	2B4	Ly49D	KLRG1
Percent[1]	D^b	3.6 (0.5)	80 (7)	91 (3)	97 (3)	93 (3)	48 (4)	48 (9)[2]
	L^d	3.3 (0.8)	77 (10)	90 (4)	93 (6)	85 (3)	49 (11)	58 (8)[3]
	K^b	3.2 (0.8)	76 (10)	92 (3)	97 (3)	92 (5)	47 (5)	51 (9)[2]
	D^d	2.9 (0.8)	78 (7)	93 (2)	97 (3)	92 (2)	46 (3)	63 (5)[2]
	MHC^-	3.9 (1.1)	74 (7)	91 (2)	92 (8)	92 (3)	37 (7)	37 (11)
MFI[4]	D^b	111 (14)	117 (23)	100 (12)	156 (44)	129 (30)[2]	97 (10)	133 (4)
	L^d	114 (8)	125 (24)	102 (14)	102 (31)	129 (44)	78 (8)[3]	153 (9)[2]
	K^b	101 (15)	99 (15)	109 (23)	139 (44)	116 (24)	100 (8)	152 (28)[3]
	D^d	101 (20)	107 (30)	108 (17)	138 (36)	127 (41)	90 (8)[2]	171 (18)[3]

[1]Percentage of $NK1.1^+CD3^-$ cells, except for NK1.1, that shows percentage of $NK1.1^+CD3^-$ cells in whole spleen. Numbers in parentheses indicate SD.
[2]$P < 0.05$ compared with MHC^- mice.
[3]$P < 0.01$ compared with MHC^- mice.
[4]Mean fluorescence intensity in percentage of MHC^- mice. Numbers in parentheses indicate SD.

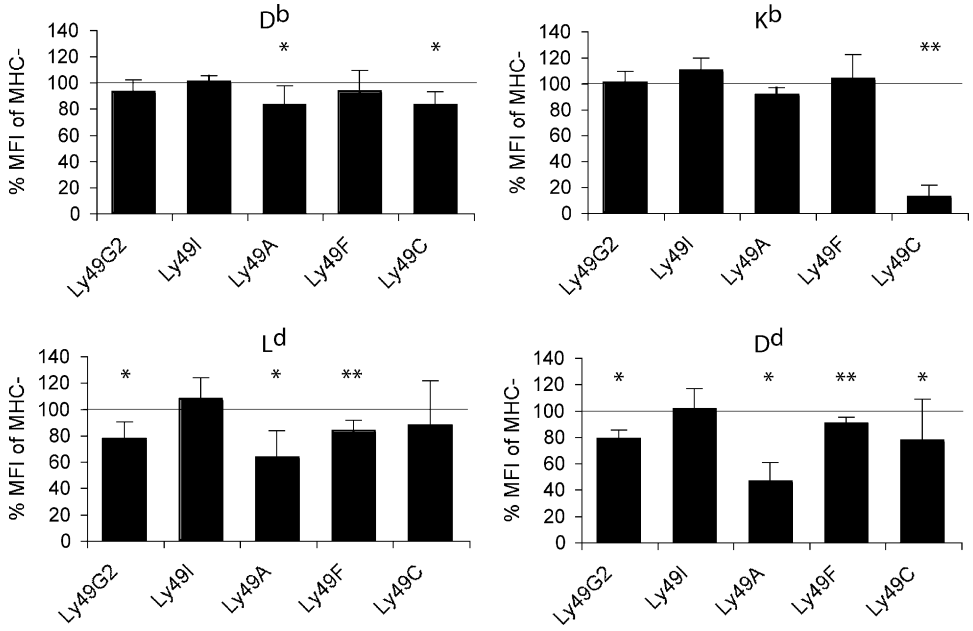

Figure 5. Unique expression patterns of Ly49 receptors in mice with single MHC class I molecules. The surface levels of Ly49G2, -I, -A, -F, and -C were measured on NK1.1$^+$CD3$^-$ cells from mice expressing no MHC class I (MHC$^-$), D^b, K^b, L^d, or D^d. The expression levels are expressed as percent of median fluorescence intensity (MFI) compared with MHC$^-$ mice. The bars show averages of four to seven experiments with one to three mice in each experiment. Error bars show SD. *, $P < 0.05$; **, $P < 0.01$.

this paper) can therefore be regarded as an imprint of previous interactions with cognate MHC class I ligands (24–27). Using this imprint, we screened for potential Ly49–MHC class I interactions in the single MHC class I mice. All single MHC class I mice showed a unique expression pattern of Ly49 receptors (Fig. 5). The receptors that showed a statistically significant down-regulation compared with MHC$^-$ mice (indicated by asterisks in the figure) were Ly49A and Ly49C in D^b single mice, Ly49C in K^b single mice, Ly49G2, Ly49A, and Ly49F in L^d single mice, and Ly49G2, Ly49A, Ly49F, and Ly49C in D^d single mice (Fig. 5). An important first conclusion from this analysis is that the educating impact by a single MHC class I molecule cannot be predicted from the number of NK cells carrying at least one significantly down-regulated Ly49 receptor. For example, both D^b single mice and L^d single mice contained larger numbers of NK cells with any down-regulated Ly49 receptors than did K^b single mice (46% and 63% vs. 38%) despite showing a lower educating impact and displaying a weak rejection potential against MHC$^-$ cells (Table III and Fig. 1). The inhibitory receptor NKG2A/CD94 recognizes the MHC class Ib molecule Qa-1^b containing a peptide derived from the leader sequence of some MHC class I molecules (4, 19, 28), in our case D^b, L^d, and D^d, but not K^b (29, 30). NKG2A is therefore a potential inhibitory receptor in all our single MHC class I mice except K^b single mice. The number of NKG2A$^+$ NK cells did not differ between the single MHC class I mice (Table III). The inclusion of NKG2A$^+$ NK cells in our calculations (31) further strengthened our conclusion that simply assessing the number of NK cells expressing an inhibitory receptor directly or indirectly recognizing self MHC class I is not sufficient to predict educating impact (Table III).

A second conclusion from the analysis of Ly49 expression was that the extent of down-regulation of individual Ly49 receptors differed among the mice (Fig. 5). For example, down-regulation of Ly49C was strong in K^b single mice, intermediate in D^d single mice, and weak in D^b single mice (Fig. 5). Another receptor that showed different degrees of down-regulation was Ly49A, which was differentially down-regulated in D^d, L^d, and D^b single mice and was not down-regulated at all in K^b single mice (Fig. 5). We have previously suggested that the degree of Ly49 down-regulation reflects the strength of MHC class I–Ly49 interactions in vivo (24), and we therefore considered the possibility that quantitative differences could contribute to the educating impact of individual MHC class I molecules by triggering signals of different intensities in individual NK cells. To quantify the extent of down-regulation in each mouse, we calculated a down-regulation index (DRI) for each receptor that reflected the expression level of this Ly49 receptor relative to MHC$^-$ mice (Table III). To estimate the overall influence on the Ly49 repertoire by a given MHC class I molecule, we multiplied the DRI for each individual Ly49 receptor by the frequency of NK cells expressing this receptor (to obtain a weighted DRI for this receptor) and finally calculated the sum of each individual weighted DRI (Table III, last column). For example, in L^d single mice, the weighted sum of DRI equals the DRIs of Ly49G2, Ly49A, and Ly49F times the frequency of each receptors as follows: Weighted sum of DRI = $[(23.2 \times 0.55) + (41.5 \times 0.11) + (16.9 \times 0.09)] = 18.8$. In K^b single

Table III. Frequencies of Ly49 and NKG2A receptors in single MHC class I mice

Receptor[1]	Frequency of receptor[2]	NK cells with down-regulated Ly49[3]	NK cells with down-regulated Ly49 or NKG2A[4]	DRI[5]	Weighted sum of DRI[6]
		%	%		
D^b mouse		46	68		8.5
Ly49G2	55			7.7	
Ly49A	**16**			**13.8**	
Ly49F	11			7.2	
Ly49C	**36**			**17.4**	
Ly49I	58			−0.1	
NKG2A	40			N/A	
L^d mouse		63	80		18.8
Ly49G2	**55**			**23.2**	
Ly49A	**11**			**41.5**	
Ly49F	**9**			**16.9**	
Ly49C	34			13.0	
Ly49I	59			−7.3	
NKG2A	46			N/A	
K^b mouse		38	38		33.4
Ly49G2	47			−0.2	
Ly49A	18			8.6	
Ly49F	8			−3.0	
Ly49C	**38**			**88.0**	
Ly49I	47			−9.6	
NKG2A	48			N/A	
D^d mouse		75	84		29.8
Ly49G2	**49**			**21.9**	
Ly49A	**17**			**57.5**	
Ly49F	**9**			**10.4**	
Ly49C	**36**			**23.4**	
Ly49I	54			−0.9	
NKG2A	38			N/A	

[1]Significantly down-regulated Ly49 receptors are in bold (see Fig. 5).
[2]Frequencies include all NK cells expressing the receptor, irrespective of any overlap with other receptors.
[3]The percentage of NK cells expressing any Ly49 receptor was calculated using the product rule to exclude counting NK cells expressing more than one Ly49 receptor twice. Only cells expressing a significantly down-regulated Ly49 receptors (bold) were included.
[4]The percentage of NK cells expressing either any down-regulated Ly49 receptor or NKG2A. $NKG2A^+$ NK cells were included only when the ligand is present in the host (e.g., in D^b, L^d, and D^d single mice).
[5]The DRI denotes the level of receptor down-regulation compared with the MHC^- mouse in percentage of mean fluorescence intensity.
[6]The weighted sum of DRI is the sum of individual DRI from significantly down-regulated Ly49 receptors (bold) multiplied by the indicated frequency of this receptor. The frequencies include all cells expressing the receptor, irrespective of any overlap with other receptors.

mice that had only one down-regulated receptor (Ly49C), the weighted sum of DRI equaled the DRI for Ly49C times its frequency, $88 \times 0.38 = 33.4$. When these calculations were performed for mice in all four single MHC class I groups, it became clear that these values differed among the mice, illustrating a quantitative influence of individual MHC class I molecules on the Ly49 repertoire. Interestingly, when the weighted sum of DRI was considered in parallel with the educating impact of each MHC class I molecule (Fig. 1), we found that MHC class I molecules with low educating impact (D^b and L^d) were associated with a low weighted sum of DRI, whereas strong MHC class I molecules (K^b and D^d) produced higher values. Taken together, these data lead us to hypothesize that the educating impact of a single MHC class I molecule is determined by a combination of the number of inhibitory receptors with which it interacts (directly or indirectly) and the strength of each such interaction.

DISCUSSION

The question of whether each MHC class I molecule present in a given individual will educate a separate subset of NK cells, and thus provoke a missing self response against cells

lacking that specific molecule, had not been thoroughly tested experimentally before this study. The question of whether all MHC class I molecules are equally efficient also had not been addressed in a comprehensive way. These questions are highly relevant, because mice and humans can express up to six different MHC class I molecules, all of which might be individually affected by an infection or mismatched in an allogeneic transplantation.

Of the four individual MHC class I molecules tested here, all could educate an NK cell repertoire capable of a missing self response when they were expressed as the only MHC class I molecule in the mouse. There were, however, differences in their relative NK cell–educating impact. In our experiments, K^b and D^d educated strong missing self responses, whereas D^b and L^d were less efficient in this respect.

There are at least two possible explanations for the difference in educating impact between different MHC class I molecules. The first is that MHC class I molecules with low educating impact interact with fewer NK cells than do MHC class I molecules with strong educating impact, suggesting that the strength of missing self rejection is controlled by the total number of NK cells active in missing self rejection. The following evidence from this study speaks against this idea. (a) The numbers of mature NK cells were similar in the four single MHC class I mice, as was the expression pattern of several activating receptors on NK cells. (b) The number of NK cells expressing the KLRG1 receptor, previously suggested to be a marker for NK cells active in missing self rejection (23), was not lower in mice with low rejection strength than in mice displaying good rejection. (c) The total fraction of NK cells with at least one down-regulated Ly49 receptor, with or without inclusion of NKG2A/CD94 when applicable, was not larger in mice expressing an MHC class I molecule with high educating impact than in mice expressing an MHC class I molecule with low impact.

As an alternative to a model postulating that an MHC class I molecule controls NK cell development only by influencing the number of NK cells expressing Ly49 receptors to which it directly binds, we considered a more quantitative control of NK cell education in which the strength of each interaction is important (24, 32). Such a model predicts a quantitative control of the triggering machinery in individual NK cells, in which the thresholds for activating and inhibitory signals are balanced during development to result in an optimally tuned mature NK cell. Such a relationship has been suggested by previous work (33–36) and postulates that an NK cell subject to strong inhibition via its Ly49 receptor interactions would develop into a more efficient killer of MHC^- cells. The degree of Ly49 down-regulation in mature NK cells may be an indicator of a quantitative regulation (8, 24, 37–39). In support of this model, when we included the extent of Ly49 down-regulation as a parameter in this study, we found that it varied in the same direction as the educating impact. More specifically, D^b had the lowest educating impact and also produced the lowest weighted sum of DRI. L^d had a higher educating impact and also gave rise to a higher weighted sum of DRI. A discrepancy was that, despite a lower educating impact, K^b single mice had a higher weighted DRI than D^d single mice (Table III and Fig. 1). However, D^d single mice, but not K^b single mice, also express a ligand for NKG2A, which in the case of D^d single mice increased the fraction of NK cells active in missing self rejection by 12% (Table III). This difference might explain why the educating impact of D^d may be higher than that of K^b in the single MHC class I mice. Thus, our model proposes that both qualitative (the number of different NK cell receptors engaged) and quantitative aspects (the strength of each receptor interaction) control the educating impact of a given MHC class I molecule. This model does not exclude important roles played by additional receptor systems in missing self rejections (40, 41). These receptors could act in concert with the MHC class I–controlled systems studied in this paper.

The fact that single MHC class I molecules interacted with several different Ly49 receptors is consistent with a role for multiple Ly49 subsets in missing self rejection. Previous studies in which Ly49 subsets were depleted in vivo have indeed shown that multiple Ly49 subsets may be required to achieve complete rejection of MHC mismatched bone marrow (42). Because these transplantations were made across multi-MHC mismatches, it is not possible to determine whether these subsets recognize the same or different MHC class I alleles. The prediction from our data that multiple Ly49 subsets are involved in missing self rejection against a single allelic mismatch therefore remains to be tested.

Although our data demonstrate that the introduction of a single MHC class I molecule conveyed rejection capacity against MHC^- cells as well as down-regulation of several Ly49 receptors, at least one example suggests that down-regulated levels of Ly49 molecules may be found in the absence of missing self reactivity. DL6 mice express a D^d transgene in a mosaic fashion, in which a fraction of lymphocytes express the molecule, and the residual lymphocytes do not. These mice show down-regulated levels of Ly49A but do not reject B6 grafts or their own cells that fail to express D^d. One way to account for this finding is to postulate a secondary, dominant, tolerizing effect on the NK cells imposed by the continuous presence of normal cells with a missing self phenotype (32). Thus, down-regulation of Ly49 molecules probably does not convey missing self reactivity per se but may rather serve as a marker for educated NK cells.

The situation was more complex in mice with more than one MHC class I molecule than in mice with single MHC class I molecules. The most interesting finding from this analysis was that some MHC class I molecules lost their educating impact when they were coexpressed with other MHC class I molecules, although they were effective alone or in more limited company. Fig. 6 summarizes the educating impact of all four MHC class I molecules as they were observed in mice expressing 1, 2, or 3 MHC class I molecules. From this compilation of all the data, it is clear that some MHC class I molecules were dominant in their educating impact

(i.e., they were always strong), whereas some seemed to be subdominant (i.e., they lost influence in the presence of other alleles). One possible explanation for the decrease in educating impact may be the overlapping specificities of Ly49 receptors (8, 9, 43). For example, missing D^b may not be sensed as efficiently in $K^bD^bD^d$ mice, because inhibitory signals for all D^b-binding Ly49 subsets could be accounted for by other MHC class I molecules coexpressed by target cells. This suggestion does not necessarily mean that "cross-reactive" MHC class I molecules are interchangeable with respect to NK specificity. The cross-reaction may be asymmetrical because of different affinities in the receptor–ligand interactions and further complicated by the down-regulation of receptors imposed by the presence of high-affinity ligands in the host. For example, D^b tetramers bind to NK cells from H-2^b mice via Ly49A (43). However, this tetramer binding does not occur in H-2^d mice expressing the high-affinity ligand D^d. Ly49A is down-regulated in D^d-expressing mice, presumably lowering the avidity for D^b tetramers below a critical threshold. In these mice, D^b tetramers instead bind to NK cells via Ly49C, highly expressed in H-2^d mice but not in H-2^b mice because of their expression of the high-affinity ligand K^b. This variability illustrates the flexibility in the Ly49 receptor system, with many promiscuous receptors recognizing several alleles with different affinity. Low-affinity interactions with certain ligands may be used in the absence of high-affinity ligands, thus providing some degree of missing self reactivity in mice expressing only the low-affinity ligands (such as in D^b and L^d single mice). In the presence of high-affinity ligands however, these interactions would play only a minor or no role.

A particularly interesting case was the educating impact of L^d, which became weaker in the presence of the combination of K^b and D^b but not in the presence of either of them alone (Fig. 6). One possible explanation for this effect could be that L^d interacts with two different inhibitory receptors, one that binds also K^b, and one that binds also D^b. In K^bL^d and D^bL^d mice, L^d would thus play a unique role. In $K^bD^bL^d$ mice, there would be no receptor requiring only L^d; therefore, L^d would lose its educating impact in that combination. One possible receptor that might be influenced by both L^d and D^b is CD94/NKG2A. D^b and L^d are both able to provide Qa-1^b–binding peptides. Qa-1 would thus present peptides from D^b on grafted K^bD^b cells in $K^bD^bL^d$ mice, and in this situation the lack of L^d would escape notice by NK cells. L^d may thus influence NK cell education via a combination of a weakly binding, as yet unidentified Ly49 receptor (binding also to K^b) and CD94/NKG2A, via its leader peptide binding to Qa-1^b. A shared education pathway between D^b and L^d was also suggested by the loss of D^b education in $K^bD^bL^d$ mice compared with K^bD^b mice (Fig. 6). Paradoxically, therefore, the only single MHC class I loss that would be detected in $K^bD^bL^d$ mice is loss of K^b, because other potentially educated subsets would require the simultaneous loss of both D^b and L^d to evoke a missing self response.

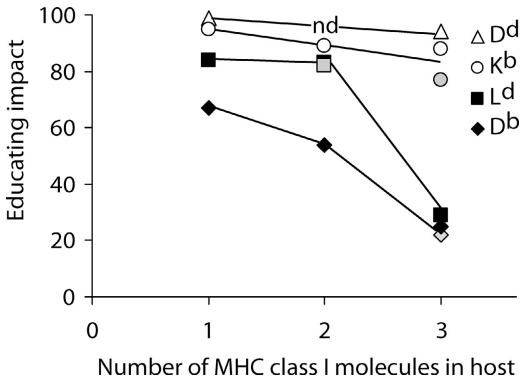

Figure 6. Summary of the educating impact of the four tested MHC class I molecules in relation to the number of additional MHC class I molecules that are coexpressed. Missing K^b and missing D^b were both tested in two different hosts with three MHC class I molecules ($K^bD^bD^d$ and $K^bD^bL^d$). The two symbols in the K^b and D^b plots indicate the different hosts with three MHC class I molecules. Similarly, missing L^d was tested in K^bL^d and D^bL^d mice, indicated by the two squares in the L^d plot with two MHC class I molecules.

We previously reported that the expression levels of Ly49G2 were unchanged in L^d transgenic B6 mice compared with nontransgenic B6 littermates, and this observation led us to suggest that L^d does not bind the Ly49G2 receptor under those conditions (15). The comparison of Ly49G2 expression between MHC^- mice and L^d single mice in the present study suggests that L^d is indeed a ligand for Ly49G2, as also reported by others from antibody-blocking experiments (44). Our preliminary data from Ly49 expression in MHC^- mice compared with single, double, and triple MHC class I mice suggest that K^b and D^b in combination down-regulate Ly49G2 to some extent and that L^d does not further impact Ly49G2 expression on top of K^b and D^b (unpublished data). This result might explain the discrepancy between our previous study and the present one. The reason why L^d does not affect Ly49G2 expression when coexpressed with K^b and D^b remains to be investigated.

The finding of a differential effect on missing self education by individual MHC class I molecules and by the same MHC on different MHC backgrounds may be relevant for antiviral defense. An MHC class I allele that is critical for CD8 T cell responses against this virus but only weakly involved in NK cell missing self recognition would provide a good target for a virus in the search for possibilities for immune escape. A virus targeting such an MHC class I allele would have an advantage over a virus that down-regulated a strong missing self ligand and thereby induced a potentially stronger NK cell response. Other areas in which our findings are relevant are the use of alloreactive NK cells in graft-versus-leukemia effects after bone marrow transplantation (45) and the involvement of KIR genes in the control of infections and autoimmune diseases (46–48). The understanding of both these situations may benefit from our data that indicate that incompatibilities between some MHC class I alleles and the corresponding inhibitory receptors may be more im-

portant than others, depending on the educating impact of the MHC class I allele in question.

MATERIALS AND METHODS

Mice. Mice were bred and maintained at the Microbiology and Tumor Biology Center (Karolinska Institutet, Stockholm, Sweden). C57BL/6 (B6) mice were originally obtained from Bomholtgård breeding and research center (Ry, Denmark). D8 mice (transgenic for D^d on B6 background) and L3 mice (transgenic for L^d on B6 background) have been described (15, 49). B6.$K^{b-/-}$, B6.$D^{b-/-}$, and B6.$K^bD^{b-/-}$ mice were generated as described (20, 50, 51). To generate mice expressing D^d as the only MHC class Ia allele, D8 mice were crossed to B6.$K^{b-/-}D^{b-/-}$ mice, the progeny were further backcrossed to B6.$K^{b-/-}D^{b-/-}$, and individuals homozygous for the K^b and D^b mutations and positive for the D^d transgene were identified using FACS analysis (BD Biosciences). A similar mating scheme was followed to generate mice expressing L^d alone. Mice expressing single MHC class I alleles were crossed to generate F_1 generations with the following genotypes: $K^b + D^d$, $D^b + D^d$, $K^b + L^d$, and $D^b + L^d$. For simplicity, the MHC class I molecules are denoted by their allelic (e.g., D^b, L^d) names only throughout the paper (Table I).

CFSE labeling and in vivo killing of CFSE-labeled cells. The method for rejection of CFSE-labeled cells has been described previously (21). In brief, spleen cells from syngeneic and test mice were stained with 0.3 and 3 μM, respectively, of CFSE (Molecular Probes, Inc). 10^7 cells of each type (syngeneic control and test population) were mixed and coinjected intravenously into the same recipients. After 18–24 h, the spleens were taken out, single-cell suspensions were made, and the relative number of cells in each CFSE population was measured by FACScan (BD Biosciences). The relative survival of $CFSE^{high}$ cells compared with $CFSE^{low}$ cells, providing a quantitative estimate of missing self rejection, was calculated as follows: (acquired number of $CFSE^{high}$ cells in sample/acquired number of $CFSE^{low}$ cells in sample)/(acquired number of $CFSE^{high}$ cells in injection mix/acquired number of $CFSE^{low}$ cells in injection mix). At least 2,000 control ($CFSE^{low}$) cells were acquired in each sample. The use of an internal control in each donor–host combination allows quantitative measurements of specific rejection in a single host. One day before injection of donor cells, recipient mice were given 2 mg tilorone analog (T-8014 or T-7514; Sigma-Aldrich) orally to augment NK activity. For NK cell depletions, 200 μg PK136 (anti-NK1.1, mouse IgG2a) or TMβ-1 (anti-IL-2Rβ, rat IgG2b) were given intraperitoneally 2 d before the experiment. PK136 and TMβ-1 were purified from hybridoma supernatants by MabTech. These two antibodies have a similar NK-depleting efficiency (unpublished data).

Calculation of educating impact of a given MHC class I molecule. The educating impact of a given MHC class I molecule in a host was defined as the efficiency by which mice expressing a particular MHC class I molecule rejected normal cells lacking this MHC class I molecule. Numerically, it takes a value of 100 minus the relative survival of cells lacking the MHC class I molecule in question.

Antibodies and FACS analysis. Splenocytes were isolated, and NK cells were enriched by nylon wool separation. Fc receptors were blocked by incubation with 2.4G2 (anti-FcγRIII). PK136-FITC and -PE (anti-NK1.1), anti-CD3-PerCP, anti-Ly49I-FITC, anti-Ly49G2-APC (4D11), anti-Ly49F-PE, anti-KLRG1-biotin (MAFA), anti-Ly49D (4E5), anti-CD16-FITC (2.4G2), anti-CD11b-PE, anti 2B4-PE, and IFN-γ–PE were purchased from BD Biosciences, and anti-NKG2D-PE (CX5) obtained from eBioscience. Anti-Ly49A (YE1/48) hybridoma was grown in our laboratory, protein G purified, and subsequently conjugated to Alexa633 (Molecular Probes, Inc.). Anti-Ly49C–biotin (4LO3311) was a gift from S. Lemieux (Université du Quebec, Quebec, Canada). To enhance the signal from Ly49C and KLRG1, the following protocol was applied. Cells were stained with anti-Ly49C– or KLRG1-biotin, washed, and stained with anti-biotin Alexa488 (mouse IgG1; Molecular Probes, Inc.), washed, and stained with anti-IgG1 Alexa488 (Molecular Probes, Inc.). The cells were washed and incubated with purified mouse IgG or mouse serum to block residual binding sites on the anti-IgG1 antibody, followed by staining for other surface markers. Flow cytometry was performed on a FACScan (BD Biosciences), and analyses were made using Cellquest software (BD Biosciences). For analysis of receptor expressions, cells were gated on lymphocytes and on $NK1.1^+CD3^-$ cells based on the forward scatter and side scatter profile.

Down-regulation index and number of cells expressing Ly49 receptors. The relative expression levels (E) of Ly49 receptors in mice expressing MHC class I compared with MHC^- mice were calculated from the fluorescence intensity (FI) as: $E = 100 \cdot (\text{median FI})/(\text{median FI in } MHC^- \text{ mice})$. A DRI for each receptor was calculated from E as: $DR = 100 - E$. A weighted DRI was obtained by multiplying the DRI for a given receptor with the fraction of NK cells expressing this receptor. The weighted sum of DRI was finally obtained by summarizing all weighted DRIs in a given single MHC class I mouse. To estimate the frequency of NK cells expressing at least one down-regulated receptor in single MHC class I mice, we applied the product rule and deduced the number of NK cells expressing several down-regulated receptors from the sum of individual Ly49 receptor frequencies (11). The same approach was used when taking NKG2A into account. It should be noted that the presence of MHC class I molecules limits the frequency of NK cells expressing multiple self-specific Ly49 receptors (11, 38, 52), and using the product rule on receptor frequencies from MHC class I expressing mice therefore probably overestimates the fraction of cells with multiple self-receptors. This caveat would, if anything, underestimate rather than overestimate the differences underlying our conclusions and thus would substantiate rather than weaken them.

Statistical analysis. To compare statistically the relative in vivo survival of cells between two recipients, a two-tailed paired Student's *t* test was used when indicated in the figure legend. The paired test was used to take into account interexperimental variation. Paired Student's *t* tests were also used to compare receptor expression levels in single MHC class I mice with those in MHC^- mice.

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