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Modulation of Ly49A receptors on mature cells to changes in major histocompatibility complex class I molecules

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SUMMARY

The expression of murine Ly49 receptors on natural killer (NK) cells and NK1.1$^+$ T cells is believed to prevent these cells from responding against normal self-tissues. In this report we investigated whether the expression level of Ly49A was fixed on mature cells or if it could be adapted as the major histocompatibility complex (MHC) class I environment changed *in vivo*. By transferring peripheral T cells from Ly49A transgenic mice into BALB/c *nude/nude* and B6 *nude/nude* mice, we demonstrated that mature cells modulate their Ly49A receptor expression relative to the *in vivo* MHC class I environment. These results indicated that the expression of the inhibitory Ly49A receptor is not permanently fixed during a maturation and/or education process but rather is adapted to MHC class I changes on the surrounding cells.

INTRODUCTION

Natural killer (NK) cells have a role in bone marrow graft rejection and can kill a variety of virally infected cells as well as tumour cells.[1,2] In addition, NK cells are an important bridge between the natural and adaptive immune systems owing to their ability to secrete various immunomodulatory cytokines. The functions of NK cells are inhibited by major histocompatibility complex (MHC) class I molecules present on the surface of normal cells, and the expression of self-MHC class I molecules is necessary for complete inhibition of NK-cell responses *in vivo*.[3–6] NK cells are inhibited when MHC class I molecules bind to inhibitory receptors of the Ly49 or KIR families. The 'missing self' theory proposes that NK cells scan potential targets for the presence of MHC class I molecules and use these Ly49 and KIR inhibitory receptors to prevent damage to normal self-tissue.[1,7]

The 'education' of murine NK cells to self-MHC class I molecules appears to involve the modulation or calibration of Ly49 receptors to the particular type and number of MHC class I molecules expressed. Ly49A binds to H-2D^d and H-2D^k, Ly49C binds to H-2K^b and H-2^d, and Ly49G2 binds to H-2D^d.[8–10] The expression of Ly49A, Ly49C and Ly49G2 have been demonstrated to be altered in mice that express MHC class I molecules that bind to them.[11–20] An analogous observation in humans has been reported in which analysis of NK cells from patients deficient in MHC class I expression indicated that their NK cells expressed increased levels of the receptor CD94-NKG2A, a human receptor that is structurally homologous to the murine Ly49 members.[21] When NK cells that expressed different levels of Ly49A were examined, a quantitative aspect to receptor-mediated inhibition was shown, in which NK cells with lower expression of Ly49A required higher expression of its ligand, H-2D^d, on target cells to prevent NK-cell lysis.[13,22] The receptor-calibration model proposes that the modulation of NK-cell receptors allows NK cells to be better able to distinguish between self and altered or missing self-MHC class I molecules.[23] Delineating the regulation of murine Ly49 receptors is important in order to comprehend how NK cells maintain tolerance to self-MHC class I molecules.

Ly49 receptors are also expressed on NK1.1$^+$ T (NK-T) cells.[24,25] These NK-T cells express a limited T-cell receptor repertoire and have been recently implicated as important regulatory cells in autoimmune model systems.[26,27] The importance of Ly49 receptors on these cells remains unclear, but NK-type inhibitory receptors have been demonstrated to inhibit T-cell activity in human[28] and murine[29] systems, thus suggesting a similar inhibitory role in the NK-T cells.

Recent data have shown that when NK cells are removed from their *in vivo* environment, their target cell specificity may change,[30,31] suggesting that maintaining NK-cell tolerance could be an ongoing process. Down-regulation of Ly49A receptors, as a result of the expression of its MHC class I ligand, has been shown to alter the function of Ly49A$^+$ NK cells,[13,22] and the extent of Ly49 expression may depend on the specific location of the NK cells.[18] One theory to account for these data is that cells may be able to continually modulate their Ly49 receptors owing to changes in the MHC class I environment throughout the lifetime of the cell. In this report we describe experiments that tested this hypothesis directly by *in vivo* transfer experiments.

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MATERIALS AND METHODS

Animals

C57BL/6 (B6; H-2^b), BALB/c (H-2^d), CB6F1 (BALB/c × B6; H-$2^{b/d}$) mice and B6 *nude/nude*, BALB/c *nude/nude* were purchased from Bomholtgård (Ry, Denmark). D8 mice and B649A transgenic mice were bred and maintained at the animal facility at Umeå University. B649A transgenic mice were produced using a 14-kb fragment containing the CD2 expression cassette and a Ly49A cDNA that was injected into (C57BL/6 × (CBA × C57BL/6)F_1)BC1 fertilized eggs, as described previously.[18] The original founder line (line 9) was backcrossed, for at least five additional generations to C57BL/6, prior to these experiments.

Antibodies

Antibodies used were: 2.4G2 (anti-FcRγ), 536.72 (anti-CD8), TIB207 (anti-CD4), HB102 (anti-H-$2D^d$), biotin (B)-conjugated YE1/48 (anti-Ly49A), B-5E6 (anti-Ly49C/I), B-4D11 (anti-Ly49G2), fluorescein isothiocyanate (FITC)-conjugated PK136 (anti-NK1.1) and FITC-conjugated HB102 (anti-H-$2D^d$). Phycoerythrin (PE)-conjugated anti-CD3ε was purchased from PharMingen (La Jolla, CA). RED670-conjugated streptavidin was purchased from Life Technologies (Täby, Sweden).

Flow cytometry

To inhibit non-specific binding of antibodies to FcRγ, spleen cells and liver cells (both depleted of erythrocytes), or lymph node cells, were incubated with anti-FcRγ for 20 min at 4° prior to staining with specific antibodies. Staining was carried out as previously described.[18] Briefly, cells (10^6) were incubated with primary antibodies for 30 min at 4°. After washing with phosphate-buffered saline (PBS) containing 1% fetal calf serum (FCS) (staining buffer), cells were incubated with secondary antibodies for another 30 min at 4°. After washing to remove unbound secondary antibodies, cells were resuspended in PBS and analysed using a FACScalibur (Becton Dickinson). Cells (25 000–200 000) were analysed using three fluorochromes and geometric mean values were determined.

Isolation of lymphocytes from liver

Livers were perfused with PBS to remove as much blood as possible. Lobes from the livers were cut into small pieces and single-cell suspensions were made by mechanical disruption through steel mesh. Cells were washed twice in 25 ml of PBS and lymphocytes were isolated by centrifugation over Percoll® gradient (Pharmacia-Upjohn, Uppsala, Sweden). Remaining contaminating erythrocytes were lysed by treatment with ammonium chloride solution.

Generation of bone marrow chimeras

Recipient mice (B6 and CB6F1) were given acid water (pH 2·5) 5 days prior to lethal γ-irradiation (8·5 Gy, Alcyon II ^{60}Co source) to prevent sepsis caused by whole body irradiation. To avoid NK cell-mediated rejection of bone marrow cells (BMC), 0·2 mg anti-NK1.1 antibodies were injected intraperitoneally (i.p.) 2 days before BMC transplantation. Five hours after γ-irradiation, mice were injected intravenously (i.v.) with 7·5–10 × 10^6 BMC from donor mice. The BMC were preincubated with anti-CD4 and anti-CD8 antibodies and treated with baby rabbit complement to deplete mature T cells before transplantation.

Two-stage transfer experimental protocol

Seven weeks after BMC transplantation, spleens were removed, single-cell suspensions were prepared and passed over a nylon wool column to enrich for T cells.[32] The nylon wool non-adherent (NWNA) spleen cells from the CB6F1 BM chimeras were incubated with HB102 supernatant (anti-H-$2D^d$) for 30 min at 4° and treated with baby rabbit complement (Cedarlane, Hornsby, ON, Canada) to remove potential contaminating host cells. Cells from B6 chimeras were treated with rabbit complement only. Secondary recipient mice (B6 *nude/nude* and BALB/c *nude/nude*) were given acid water 5 days prior to γ-irradiation (5 Gy). Five hours after irradiation, the secondary recipient mice were injected i.v. with 2–2·5 × 10^6 complement-treated NWNA splenocytes from the BMC chimeras and 5 × 10^6 syngenic BMC. Control B6 *nude/nude* mice were injected only with syngenic BMC. Six weeks after transplantation, spleen and lymph nodes (LN) were removed, and single-cell suspensions were prepared, stained and analysed by flow cytometry.

Single stage-transplant of NWNA spleen cells into nude mice

Recipient mice (B6 *nude/nude* and BALB/c *nude/nude*) were injected i.v. with 3 × 10^6 NWNA spleen cells from donor mice. Donor mice used were B649A (H-2^b) and BALB/c × B649A (H-$2^{b/d}$). Between 5 and 7 weeks after transplantation, the spleen and lymph nodes were removed, single-cell suspensions were prepared and analysed by flow cytometry.

Statistics

Data were analysed using the paired Student's *t*-test. Differences were considered significant when *P*-values were less than 0·05.

RESULTS

Ly49 receptor expression and extent of MHC class I H-$2D^d$-induced down-regulation is dependent upon the location of NK cells and NK-T cells

We and others have shown that Ly49 receptors on NK cells are down-regulated in mice that express MHC class I ligands for those Ly49 receptors.[11–20] As NK-T cells also express Ly49 receptors, we examined whether Ly49 receptors were also down-regulated on NK-T cells (Fig. 1). Similar to that observed with NK cells, NK-T cells down-regulated Ly49A and Ly49G2 in B6 mice that expressed an H-$2D^d$ transgene (D8 mice) compared with wild-type B6 mice. A greater down-regulation was observed on NK-T cells in the thymus than those in the liver of D8 mice (Table 1). As we have seen in Ly49A transgenic mice,[18] the expression of Ly49 receptors is higher on NK-T cells in the thymus than in the periphery, with the exception of Ly49G2, which is expressed at a lower level in thymus than in liver NK-T cells in D8 mice. Like the Ly49 receptor regulation observed on NK cells, Ly49A expression on NK-T cells was down-regulated to a greater extent than Ly49G2, in the presence of H-$2D^d$. There was no significant change in the expression of Ly49C and Ly49I receptors on NK-T cells between B6 and D8 mice. As reported earlier, we measured small differences between the expression

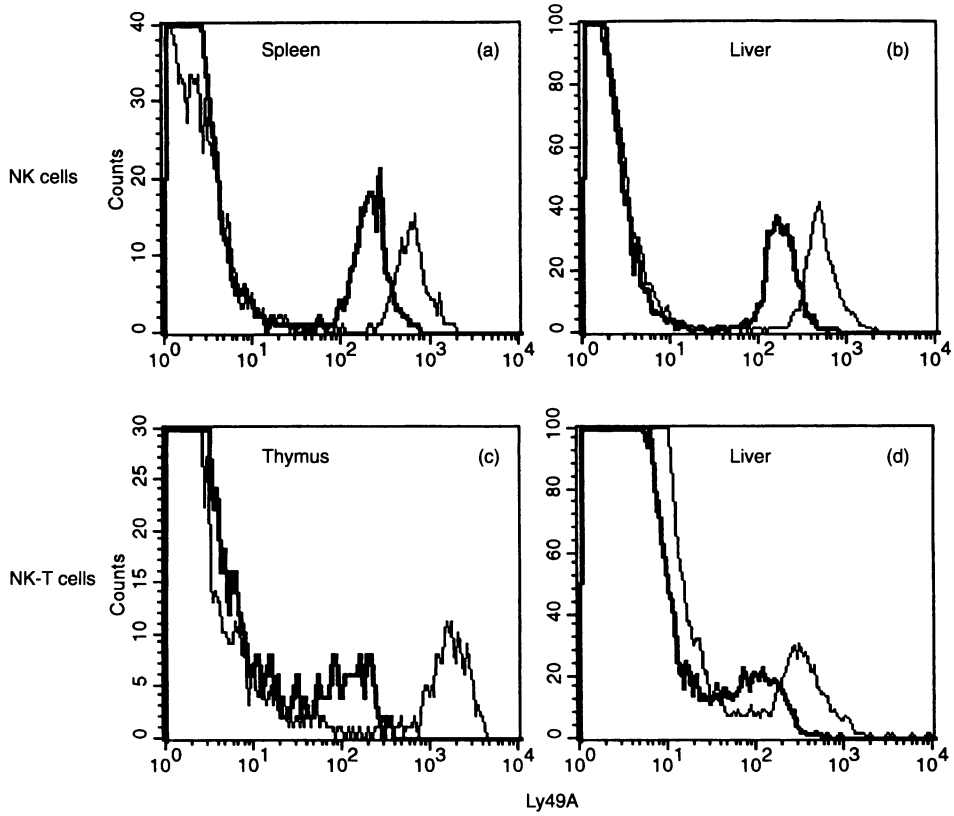

Figure 1. Ly49A receptors are down-regulated on NK and NK-T cells in D8 mice. The expression levels of endogenous Ly49A in the spleen (a) thymus (c) and liver (b, d) on NK cells (a and b; $NK1.1^+$ $CD3^-$ cells) and NK-T cells (c and d; $NK1.1^+$ $CD3^+$ cells) are shown for D8 mice (thick line) and B6 mice (thin line). These histograms are derived from representative mice.

Table 1. Expression of Ly49 receptors on NK cells and NK-T cells in different organs from B6 and D8 mice

	Ly49A expression			Ly49C/I expression			Ly49G2 expression		
	Mouse strain			Mouse strain			Mouse strain		
	B6	D8	%	B6	D8	%	B6	D8	%
$NK1.1^+$, $CD3^-$									
Spleen	312*	113	36†	115	102	88	159	123	77
Liver	308	100	32	97	84	86	131	103	79
$NK1.1^+$, $CD3^+$									
Thymus	384‡	69	18§	132§	142§	107	138	51‡	37¶
Liver	146	51	35¶	70	72	103	121	95	79**

*Numbers represent average geometric mean fluorescence intensity of Ly49 expression on $NK1.1^+$, $CD3^-$ cells or on $NK1.1^+$, $CD3^+$ cells.

†Numbers represent Ly49 expression in D8 mice as a percentage of Ly49 expression in B6 mice.

‡Expression on thymocytes was different from that on liver cells ($P<0.05$).

§Expression on thymocytes was different from that on liver cells ($P<0.005$).

¶Expression in B6 mice was different from that in D8 mice ($P<0.005$).

**Expression in B6 mice was different from that in D8 mice ($P<0.05$).

of Ly49 receptors on NK cells in the spleen and those in the liver.[18] Taken together, these data indicate that Ly49 receptors are regulated in a similar manner on NK-T cells, as previously seen on NK cells. The data also suggest that the expression and down-regulation of the Ly49 receptors depends on the organ where the $Ly49^+$ receptor cells reside.

Ly49A receptor expression is adaptable on mature cells and can be regulated by both haematopoietic and non-haematopoietic cells

To test the hypothesis that Ly49 receptors can be altered on mature cells relative to the *in vivo* environment, we used a two-stage transfer experiment (Fig. 2, Table 2). Bone marrow cells from B649A transgenic mice were transplanted into either B6 ($H\text{-}2^b$) or (BALB/c × B6)F_1 ($H\text{-}2^{d/b}$) mice. The T cells that develop in the F_1 mice will be tolerant to both BALB/c ($H\text{-}2^d$) and B6 ($H\text{-}2^b$) MHC class I molecules, although they will only express $H\text{-}2^b$ MHC class I molecules themselves. The level of Ly49A expression on the $CD3^+$ cells was significantly down-regulated in the F_1 recipient mice that expressed an MHC ligand for Ly49A compared with B6 recipient mice (Fig. 2c). After 7 weeks, NWNA spleen cells were isolated and treated with anti-$H\text{-}2D^d$ antibody and complement to remove any potential remaining endogenous $H\text{-}2D^d$-bearing F_1 cells. Although there were no detectable cells of F_1 origin after separation over the nylon wool column, the cells were treated with antibody and complement to ensure the removal of any rare F_1 cells that may have been undetectable by flow

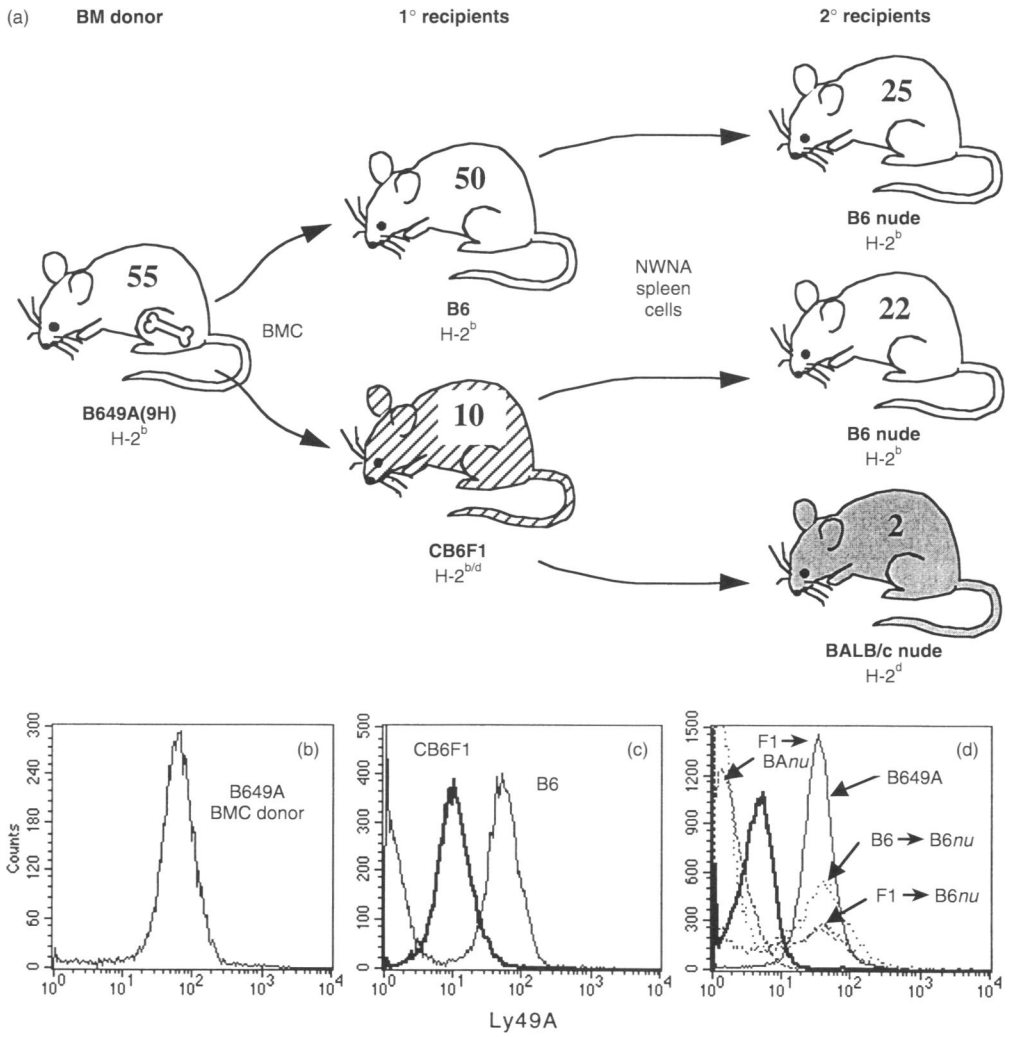

Figure 2. Modulation of Ly49A receptor on mature cells upon transfer into different MHC haplotypes. The diagram (a) illustrates transfer of bone marrow cells in the first stage followed by transfer of NWNA splenic cells into nude mice of H-2^b or H-2^d haplotypes in the second stage. Values indicated within the mice are the geometric mean fluorescence intensity of Ly49A expression of $CD3^+$ spleen cells. The expression of Ly49A on $CD3^+$ cells from the BMC donor (b), from B6 (thin line) and $CB6F_1$ (dark line) recipients after first-stage transfer (c), and from mice receiving NWNA splenocytes in the second transfer (d); B649A transgenic (thin line); Ly49A transgenic F_1 (thick line) (no arrow); $CB6F_1 \rightarrow$B6 *nude/nude* ($F_1 \rightarrow$B6*nu*, dashed line); B6→B6 *nude/nude* (B6→B6*nu*, dotted line); $F_1 \rightarrow$BALB/c *nude/nude* ($F_1 \rightarrow$BA*nu*, broken line). Data from B6 *nude/nude* recipients are shown as histograms, based on a scale from 0 to 750 counts. B6*nu*=C57BL/6 *nude/nude*, and BA*nu*=BALB/c *nude/nude*.

cytometry. These NWNA spleen cells were transplanted into either B6 *nude/nude* mice or BALB/c *nude/nude* mice in the second stage. As nude mice lack a thymus, no new T cells are able to develop. After a period of 6 weeks, to allow the transferred T cells to populate the lymphoid organs,[33] the $CD3^+$, H-$2D^d$- spleen cells were examined for their expression of Ly49A (Fig. 2d). The cells transferred into the B6 (H-2^b) nude mice had increased their Ly49A expression to a similar extent as that observed in the B6 to B6 nude control transfer, while the cells from the same population transferred into the BALB/c (H-2^d) nude mice had down-regulated their expression of Ly49A to an even lower level than observed on F_1 mice. Similar data were obtained when $CD3^+$ lymph node cells were examined (data not shown). Thus, mature cells can modulate their expression of Ly49 receptors, depending on the *in vivo* MHC environment.

We also used a single-stage transfer experiment to test the ability of Ly49 receptors to adapt to a new MHC environment *in vivo*. NWNA spleen cells were transferred from B649A (H-2^b) mice or (BALB/c × B649A)F1 (H-$2^{d/b}$) mice to BALB/c *nude/nude* or B6 *nude/nude* mice. Between 5 and 7 weeks post-transfer, lymph node cells were analysed for their expression of Ly49A (Table 3). The cells transferred into BALB/c nude mice continued to express low levels of Ly49A. The expression of Ly49A increased on cells transferred into B6 nude mice, but the Ly49A expression did not reach the level found on B649A to B6 nude chimeric $CD3^+$ lymph node cells. Although the host cells expressed no MHC class I ligands for Ly49A, the transferred donor cells themselves expressed H-$2D^d$, which appeared to regulate the expression of Ly49 receptors. Similar data were obtained when $CD3^+$ splenic cells were examined (data not shown). These data confirm earlier findings that

Table 2. Expression of Ly49A on spleen cells from the two-stage transfer experiments

	Experiment 1*		Experiment 2†	
	Ly49A	n‡	Ly49A	n
BMC donor				
B649A	11·3§		55·5	
1° Recipients				
B649→B6°	15·9		50·0	
B649A→CB6F1°	5·3		10·0	
2° Recipients				
B6°→B6 nude	13·8	4	25·0	3
F_1°→B6 nude	14·8	4	22·0	3
F_1°→BALB/c nude	6·6	2	2·0	2

*B649A Tg heterozygote used as a BMC donor.

†B649A Tg homozygote used as a BMC donor.

‡n = number of mice analysed in each group.

§Numbers represent average geometric mean fluorescence intensity of Ly49A expression on $CD3^+$ spleen cells.

Table 3. Expression of Ly49A on lymph node cells from the single-stage transfer experiment

	Ly49A expression	n*	%
B649A	26·2 (10)†	4	100‡
(BALB/c × B649A)F_1	4·8 (0·5)	4	18
F_1→BALB/c nude	4·6 (0·6)	5	18
F_1→B6 nude	10·9 (2·5)	7	42
B649A→B6 nude	29·3 (6·5)	6	112

*n, number of mice analysed in each group.

†Numbers represent average geometric mean fluorescence intensity of Ly49A expression on $CD3^+$ lymph node cells. Numbers in brackets are standard deviations.

‡Numbers represent Ly49A expression as a percentage of B649A values.

haematopoietic cells themselves can regulate Ly49A expression,[12,18] and like the cells in the double-transfer experiment, these transferred cells were able to modulate their expression of Ly49A to different MHC environments *in vivo*.

DISCUSSION

In this report, we have examined the regulation of Ly49 expression on NK-T cells and demonstrated, using cell transfer protocols and Ly49A transgenic mice, that the expression of Ly49 receptors on mature cells can be adapted to changes in the MHC class I environment *in vivo*. Two recent reports demonstrate that NK cells, which are tolerant *in vivo*, can kill the same target cells upon culture with recombinant interleukin-2 (rIL-2) *in vitro*.[30,31] Our previous report suggested that Ly49 receptor expression may adapt on mature cells, depending on the *in vivo* microenvironment where they reside.[18] Thus it is unlikely that tolerance of NK cells to self-tissue and expression of their repertoire of inhibitory Ly49 receptors is only the result of a selection process during NK-cell maturation. One prediction of an adaptation model is that exposure of mature $Ly49A^+$ cells to different MHC class I environments should readjust their expression of Ly49A receptors. We took advantage of Ly49A transgenic mice where all T cells express Ly49A. Using a two-stage transfer protocol, T cells ($H\text{-}2^b$) that developed in (BALB/c × B6) F_1 mice ($H\text{-}2^{d/b}$) expressed low levels of Ly49A, but upon subsequent transfer of mature T cells to BALB/c nude or B6 nude mice, the cells changed their Ly49A expression to low or high levels, respectively.

The adaptation of antigen-specific receptors to the peripheral environments has been reported for T-cell receptors and immunoglobulin.[34–37] In one report,[34] the expression of T-cell receptors specific for paternal antigen on maternal T cells was reduced during pregnancy when paternal antigen was expressed by the fetus. In addition, the T-cell receptor levels increased after delivery, even in thymectomized female animals. In a similar manner, cells appear to be capable of modulating their expression of NK inhibitory receptors to particular physiological conditions.

It may seem paradoxical that Ly49 receptors are down-regulated in the presence of their MHC ligands and up-regulated when those MHC ligands are not present. The triggering of NK cells is thought to be a delicate balance between signals received by activating receptors and signals received by inhibitory receptors. Thus, to increase the likelihood that a given signal will activate a NK cell, it is possible to increase the expression of activating receptors or to decrease the expression of inhibitory receptors. The receptor-calibration model describes how the down-regulation of Ly49 receptors to self-MHC class I may produce effector cells that are more sensitive to small changes in self-MHC class I molecules.[23]

The continual adjustment or calibration of Ly49 receptors *in vivo* has important implications for cellular function. It has been observed that even a twofold decrease in Ly49A expression can alter the ability of killer cells to respond to particular target cells.[13,22] The modulation of Ly49 receptor expression may not only be a result of changes in MHC class I. Perhaps other physiological signals, or a combination of the two, may alter Ly49 receptor expression. The adjustment of Ly49 receptors would allow cells to adapt their Ly49 receptors to the conditions within the tissue where they become residents. In this manner, the cells would be readily prepared to recognize small changes in MHC class I expression on nearby cells and provide a defensive response. It is intriguing to speculate on the biological significance of these observations in the context of various situations –tumour growth, chronic viral infection and pregnancy – where conditions exist that may alter the expression of inhibitory receptors and probably their biological function. The observation that the expression of Ly49 receptors can change on mature cells indicates another mechanism to modify and adapt NK-T cell (and perhaps NK cell) responses to changing conditions *in vivo*.

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