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ORIGINAL ARTICLE

The regulatory functions of Ly-49A, Ly-49D and Ly-49G2 on NK cells in the recognition and rejection of the alloantigen *in vivo**

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Summary

Ly-49A is an inhibitory receptor that binds H-2D^d and H-2D^k. The downregulation of Ly-49A is thought to mediate NK self tolerance *in vivo*. In this study, we analyzed the regulation of Ly-49A, D and G2 on NK cells in an *in vivo* rejection model. After injection with 1×10^8 B10.D2 spleen cells (SC) into B 10 mice, we found Ly-49A downregulated within 3 h on NK cells of B10 mice, whereas expressions of Ly-49D and G2 were augmented. To investigate effects of different expression patterns of Ly-49 receptors on NK cells, Ly-49A, D or G2-depleted B10 mice were inoculated with B10.D2 SC. NK cells from SC of Ly-49A-depleted and B10.D2 SC-injected B10 mice showed enhanced cytotoxicity to D^d-positive targets *in vitro*. Furthermore, reduced numbers of B10.D2 SC were observed in Ly-49A or G2-depleted B10 mice, whereas increased numbers of B10.D2 SC were observed in Ly-49D-depleted B10 mice after inoculation with B10.D2 SC *in vivo*. These findings indicated that the downregulation of Ly-49A and the augmentation of Ly-49D expression may mediate NK cells to recognize and kill D^d antigen efficiently. In conclusion, each Ly-49 isoform may play independent roles in the regulation of activation or inhibition on NK cells.

Introduction

NK cells have the ability to kill the tumor cells and the infected cells that have lost or express insufficient amount of MHC class I molecules [1], and mediate the acute rejection of the bone marrow allograft or 'hybrid resistance' between F0-F1 mice [2]. It is becoming clear that the NK cells are able to recognize the class I molecule of target cells via 2 families of cell surface receptors, CD94/NKG2A and Ly-49 families [3,4]. Ly-49 family of receptors is composed of C-lectin type II membrane glycoproteins [3–5]. Some of the members of Ly-49 families are inhibitory receptors and have been shown to possess the VxYxxV immunoreceptor tyrosine inhibition motif (ITIM) in their cytoplasmic tail[5,6]. On the other hand, Ly-49D and Ly-49H members of Ly49 families, which lack ITIM motif, function to activate NK cells to lyze the target cells [7,8]. *In vitro* studies, inhibitory receptors have been shown to function dominantly if they are co-expressed on NK cells with activation receptors [9,10].

Ly-49A receptor inhibits the NK cell lyses of target cells which express H-2D^d and H-2D^k, and is downregulated in the H-2D^d and H-2D^k strain mice[11,12]. Ly-49G2 is the inhibitory receptor which recognizes H-2D^d and H-2L^d and, similarly to Ly-49A, is downregulated in H-2^d mice[12,13]. This downregulation is dependent on the presence of MHC class 1 [11,14,15] and occurs post-translationally, as it is independent of receptor mRNA transcript levels [16]. Referred to as 'receptor-calibration',

it is believed that the downregulation of inhibitory receptors directed toward self-MHC enables the NK cell to detect small alterations in MHC class I expression [17].

Ly-49D and Ly-49H have been reported to be activation receptors of NK cells [7,8]. Ly-49D recognizes H-2D^d, along with the xenogeneic antigen of RT1^{lv1} and RT1^l haplotypes and functions to mediate target cell killing [18]. The rejection of D8 BHC in H-2bmice can be reversed by the administration of anti-Ly49D/A mAb and suggesting that the Ly-49D-positive NK cells are responsible for the rejection of D^d [13].

In the present study, we analyzed the regulation of Ly-49A, Ly-49D and Ly-49G2 expression on NK cells using a transplant rejection model that involved the injection of B10.D2 SC into B10 mice. We found the downregulation of Ly-49A after the injection with H-2D^d-positive allogeneic cells. Thus, controversy to the previous 'receptor-calibration' which was mainly insisted the co-relation between downregulation and self- or allogeneic tolerance [19,20], the downregulation was also found during rejection of allogeneic cells. However, NK cells still received efficient inhibitory signaling via these receptors despite the downregulation during the rejection of H-2D^d-positive allogeneic cells. We hypothesized that the expression of Ly-49A may be consistently maintained on NK cells depending on its surrounding antigens so as not to receive exceeding negative signaling. We also found the augmentation of Ly-49D and Ly-49G2 during rejection of allogeneic cells which were regulated in duration of exposure and dose of antigen. However, the time course of the regulation was much slower comparing to Ly-49A. Thus, NK cell Ly-49 receptor family members were more closely regulated on NK cells in our model.

Materials and methods

Mice

Inbred mice of C57BL/10 SnSlc (B10; H-2^b) and B10.D2 SnSlc (B10.D2; H-2^d) strains were obtained from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan). These mice were used at 8–12 weeks of age.

Tumors and cell lines

YAC-1 is a Moloney leukemia virus-induced lymphoma of A/Sn origin (H-2^a). RL♂1 is a radiation-induced leukemia in BALB/c mice (H-2^d). P815 is a methylcholanthrene-induced mastocytoma in DBA/2 mice (H-2^d). All animals received humane care in compliance with both the Guidelines for Animal Experiments in Kyushu University and the Law (No. 105) and Notification (No. 6) of the Japanese government.

Cell preparation

Mice were killed by decapitation. The spleens were collected and disrupted in the RPMI 1640 medium (GIBCO, Grand Island, NY, USA) by pressing spleen fragments between two-glass slides. Cell suspensions were filtered through cotton gauze and washed three times with the RPMI medium. Viable nucleated cells were counted and adjusted to 1×10^7/ml for flow cytometory analysis and 1×10^7–2×10^8/ml for the inoculation *in vivo*.

Flow cytometory analysis (FCA)

Cells were stained with fluorescein-isothiocyanate (FITC)-conjugated anti-Ly-49 monoclonal antibodies (mAb), phycoerythrin (PE)-conjugated anti-NK1.1 (PK136) mAb (PharMingen, San Diego, CA, USA), allophycocyanin (APC)-conjugated anti-mouse CD3 mAb (PharMingen), and biotinylated-anti-H-2D^d mAb (PharMingen) for 30 min at 4 °C and washed twice. FITC-conjugated ant-Ly-49A (YEL 48; PharMingen), Ly-49D (4E5; PharMingen) and Ly-49G2 (4D11; PharMingen) mAb were used to detect Ly49 expression. To block non-specific FcγR binding of labeled mAbs, 20 μl of undiluted culture supernatant of 2.4G2 (rat anti-mouse FcγR mAb) was added to the first incubation. Cell bound biotinylated mAb was detected with streptavidin-Cy-Chrome (SA-Cy-Chrome; PharMingen). All data were analyzed with a FACSCalibur (Becton Dickinson, Sunnyvale, CA, USA), and analyzed with CELL QUEST software (Becton Dickinson).

To determine the percentage of each Ly-49 expression on NK cells, 3000–5000 gated H-2D^d-negative CD3-negative NK1.1-positive (NK) cells were collected. Also, data were collected as the median fluorescence channel of percentage-positive cells. The relative median fluorescence intensity (MFI) was calculated by dividing the observed MFI of the peak of cells staining with mAbs for each analysis by the observed MFI of the same cell population from untreated B10 and multiplying the quotient by 100%. The number of H-2D^d-positive allogeneic cells was calculated from the percentage and total spleen cells number.

Abs utilized for depletion *in vivo*

B10 mice were injected with 50 μg of mAb YEL48 (anti-Ly-49A), 4E5 (Ly-49D) or 4D11 (Ly-49G2) which were kind gift from Dr William J. Murphy (SAIC-Frederick Cancer Research and Development Center, Frederick, MD, USA) [21] or 100 μg of mAb PK 136 (anti-NK1.1) 4 days before the inoculation of B10.D2 SC to deplete NK cells or its subsets. The depletion was confirmed by staining with PE-conjugated anti-NK1.1 mAb and FITC-conjugated anti-Ly49-A, anti-Ly49-D anti-Ly49-G2 mAbs,

or FITC-conjugated anti-rat IgG2a mAb for secondary staining against 4E5 and 4D11. To confirm the depletion of Ly-49A, cells were stained with FITC-conjugated anti-mouse IgG2a mAb for 30 min at 4 °C and washed twice, and then stained with PE-conjugated anti-NK1.1 mAb and used for FACS analysis. To confirm the depletion of NK cells, cells were directly stained with PE-conjugated anti-NK1.1 mAb, or stained with FITC-conjugated anti-NK1.1 mAb and used for FACS analysis. All antibodies were titered and tested for their ability to deplete NK cells and its subsets and used in this experiments.

Cytotoxic assay

Tumor cells were labeled by incubating 1×10^7 cells with 3.7 MBq of $Na_2^{51}CrO_4$ in 0.3 ml of medium for 1 h at 37 °C under 5% CO_2 in air. The cells were then washed and used as target cells. To evaluate the cytotoxicity of the NK cells, 2×10^4 labeled target cells (100 μl) were incubated with the effecter cell suspension (100 μl). After incubation for 4 h at 37 °C under 5% CO_2 in air, the supernatants (100 μl) were removed and their radioactivity was measured. The percentage of specific lysis was calculated by the following equation: $[(a - b)/(c - b)] \times 100$, where a is the radioactivity in the supernatant of target cells mixed with effecter cells, b is that in the supernatant of target cells incubated alone, and c is that in the supernatant after lysis of target cells with 1% Triton-X (Wako, Tokyo, Japan). To analyze the NK cell activity *in vivo*, whole spleen cells were used as effecter cells.

Statistics

The statistical significance of the data was determined by Student's t-test. A P-value of <0.05 was considered to be statistically significant.

Results

The regulation of the expressions of Ly49 receptors on NK cells in B10 mice after the inoculation of the B10.D2 SC

We first investigated Ly-49 expression on NK cells when NK cells reject H-2D^d-positive allogeneic cells *in vivo*. The expression of Ly-49 receptors on D^d-negative NK cells in spleens from B10 mice which had been inoculated with 1×10^8 B10.D2 SC was analyzed with a FACSCalibur. The number and the percentage of D^d-negative NK cells was the same during the time course we examined (Table 1). The inoculated allogeneic cells were detected at

Table 1. The different expressions of Ly-49A, Ly-49D and Ly-49G2 on NK cells from B10 mice inoculated with 1×10^8 B10.D2 SC.

Group	Mice	SC	Time after SC i.v.	*n*	D^{d+}cells (%)	D^d-NK1.1$^+$ cells (%) (number × 0^6)	Ly49A$^+$NK cells (%) (%MFI* B10 = 100%)	Ly-49D$^+$NK cells (%)	Ly-49G2$^+$NK cells (%)
1	B10.D2	–		3	–	–	2.1 ± 0.6†	33.5 ± 0.8†	39.4 ± 0.6
							(71.3 ± 2.1†)	(76.3 ± 1.0†)	(79.9 ± 3.2‡)
2	B10	–		3	–	3.2 ± 0.3	16.5 ± 0.4	29.2 ± 0.6	38.6 ± 1.2
						(1.8 ± 0.2)	(100.0 ± 0.1)	(100.6 ± 3.5)	(101.5 ± 3.5)
3	B10	+	3 hr	3	16.0 ± 1.6	3.1 ± 0.2§	4.4 ± 0.5†	26.7 ± 1.4‡	32.7 ± 2.1‡
						(2.2 ± 0.3§)	(71.2 ± 4.2†)	(100.6 ± 2.9§)	(95.4 ± 6.0§)
4	B10	+	6 hr	3	15.8 ± 1.2	3.5 ± 0.5§	4.5 ± 1.5†	30.3 ± 2.2§	32.3 ± 2.4‡
						(2.2 ± 0.3§)	(65.3 ± 2.1†)	(96.9 ± 0.9§)	(98.2 ± 2.7§)
5	B10	+	12 hr	3	13.4 ± 0.7	3.0 ± 0.3§	4.0 ± 1.3†	29.4 ± 0.7§	36.3 ± 0.2‡
						(2.1 ± 0.5§)	(61.1 ± 1.1†)	(102.5 ± 3.5§)	(106.5 ± 6.7§)
6	B10	+	24 hr	3	11.6 ± 1.1	3.6 ± 0.5§	3.9 ± 1.1†	31.5 ± 1.0‡	39.7 ± 0.6§
						(2.5 ± 0.4§)	(63.6 ± 0.9†)	(111.2 ± 0.9†)	(122.7 ± 6.3†)
7	B10	+	2 days	3	10.7 ± 0.3	3.1 ± 0.2§	3.9 ± 0.5†	28.7 ± 1.8§	40.9 ± 1.0§
						(2.5 ± 0.8§)	(65.6 ± 0.5†)	(121.1 ± 2.1†)	(128.6 ± 1.2†)
8	B10	+	4 days	3	0.5 ± 0.2	3.2 ± 0.2§	12.0 ± 1.0†	27.6 ± 1.1§	36.1 ± 2.4§
						(2.3 ± 0.4§)	(103.0 ± 11.1§)	(118.8 ± 6.2‡)	(135.1 ± 1.2†)
9	B10	+	1 Week	3	0.3 ± 0.1	3.4 ± 0.2§	14.0 ± 0.8†	24.7 ± 1.7‡	37.9 ± 0.5§
						(2.3 ± 0.5§)	(112.7 ± 9.6§)	(111.1 ± 4.3‡)	(110.8 ± 5.0§)
10	B10	+	2 Weeks	3	0.2 ± 0.0	2.6 ± 0.8§	15.5 ± 0.4‡	18.6 ± 0.7†	35.2 ± 1.2‡
						(1.8 ± 0.5§)	(91.6 ± 4.5‡)	(98.5 ± 3.0§)	(78.3 ± 4.6†)

*The relative medianfluorescence intensity (MFI) was calculated by dividing the observed MFI of the peak of cells staining with mAbs for each analysis by the observed MFI of the same cell population from untreated B10 and multiplying the quotient by 100%.
†$P < 0.02$ compared with the untreated B10 mice.
‡$P < 0.05$ compared with the untreated B10 mice.
§N.S. compared with the untreated B10 mice.

a level of over 10% until 2 days after the inoculation, but by 4 days after inoculation had dropped to <0.5%. We found that the expression of Ly-49A on the D^d-negative NK cells of the B10 mice was downregulated immediately after the inoculation with B10.D2 SC (Table 1, Fig. 1). This downregulation in the percentage MFI was observed as early as 3 h and persisted up to 2 days after the inoculation. Although the expression of Ly-49D in the percentage MFI was augmented from 24 h to 1 week after the inoculation, the percentage of Ly-49D-positive NK cells was almost the same during this phase, and in fact it was decreased at 2 weeks after the inoculation. Despite the fact that like Ly-49A, Ly-49G2 is also an inhibitory receptor, the regulation pattern of Ly-49G2 following inoculation was completely different from that of Ly-49A. The expression of Ly-49G2 was augmented in the percentage MFI from 24 h to 1 week after the inoculation, while the percentage of Ly-49G2-positive NK cells was almost the same during the course we examined. Thus, the expression of Ly-49A was regulated in both frequency and intensity, whereas the expression of Ly-49D and G was regulated mainly in intensity after injection of D^d-positive allogeneic cells. Furthermore, the expression pattern in the time course was different in each Ly49s.

The regulation of the expressions of Ly49 receptors in B10 mice depended on the percentage of the H-2D^d-positive allogeneic cells

Next, we inoculated with differing numbers of B10.D2 SC into B10 mice and examined the regulation of Ly-49 expression on their NK cells on day 2 (Table 2, Fig. 2). B10 mice were inoculated with different dose of allogeneic cells, such as 1×10^8 B10.D2 SC in group I, 5×10^7 in group II, 1×10^7 in group III and 5×10^6 in group IV. In group I mice, H-2D^d-positive cells were detected at a frequency of 10.9% and Ly-49A-positive NK cells were detected at a frequency of 4.5% of the percentage MFI for Ly-49A was 66.8 in group I mice. The H-2D^d-positive cells were detected at 0.8% in group III mice and Ly-49A was downregulated to a lesser degree than that observed in group I mice. Based on these findings, we correlated the level of downregulation of Ly49-A with the percentage of the H-2D^d-positive cells. Ly-49D expression, as meas-

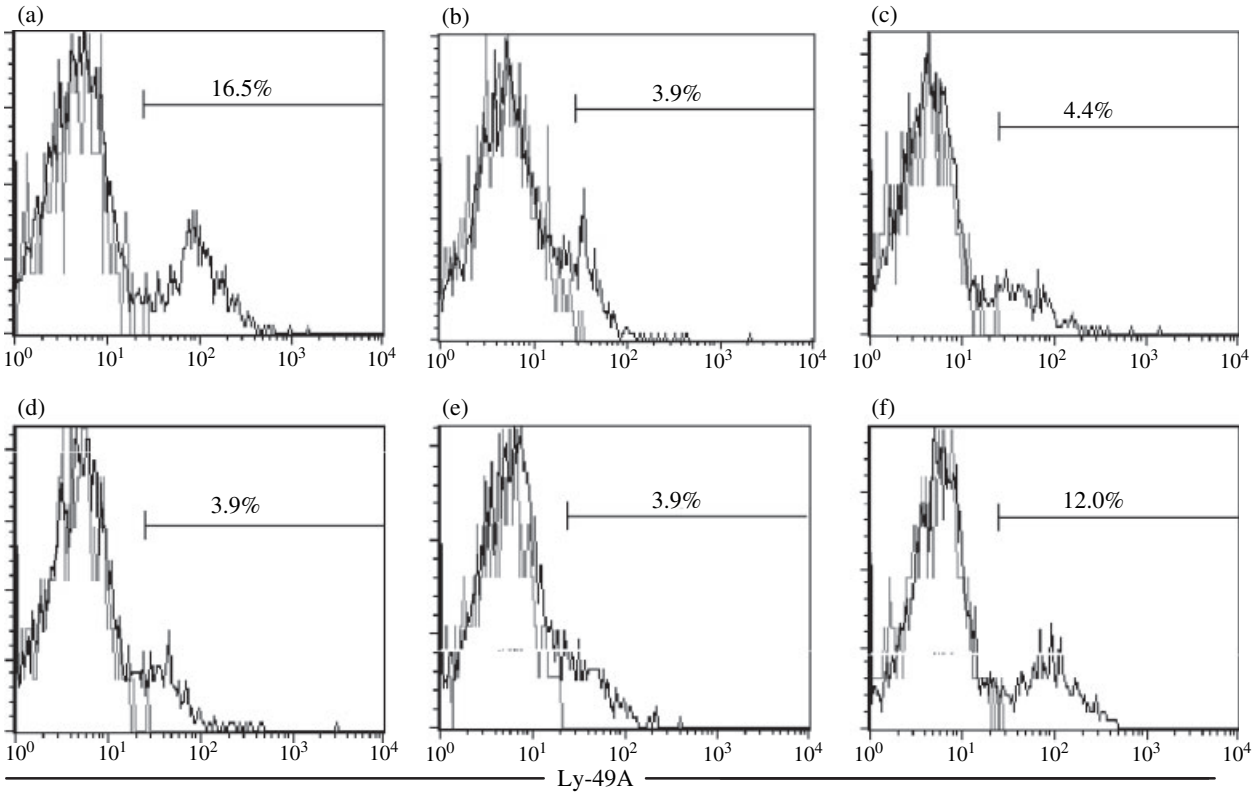


Figure 1 The downregulation of Ly-49A after the inoculation with 1×10^8 B10.D2 SC. Expressions of Ly-49A on NK cells in the spleen from (a) untreated B10 mice, (b) untreated B10.D2 mice and B10 mice which were inoculated with B10.D2 SC (c) 3 h, (d) 1 day, (e) 2 days and (f) 4 days before the analysis was analyzed by four-color fluorescence flow cytometory. The analysis gate was set on NK cells and histograms with the mean percentages of the Ly-49A$^+$ cells are shown. The data shown are from one of four experiments with representative results.

Table 2. The different expressions of Ly-49A, Ly-49D and Ly-49G2 on NK cells from B10 mice inoculated with different number of B10.D2 SC.

Group	Mice	Number of inoculated SC	n	D^{d+} cells (%)	D^{d-} NK1.1$^+$cells (%) (number $\times 10^6$)	Ly49A$^+$NK cells (%) (%MFI*: B10 = 100%)	Ly-49D$^+$NK cells (%)	Ly-49G2$^+$NK cells (%)
	B10.D2	–		–	– (–)	2.3 ± 0.5† (73.3 ± 3.1†)	46.6 ± 1.5† (76.4 ± 7.2†)	42.5 ± 1.6† (76.3 ± 4.1†)
	B10	–		–	2.9 ± 0.2 (1.8 ± 0.1)	14.9 ± 1.2 (100.1 ± 4.2)	31.7 ± 0.8 (102.2 ± 5.2)	37.9 ± 0.6 (101.5 ± 3.1)
I	B10	1×10^8	4	10.9 ± 1.1	2.5 ± 0.3§ (1.9 ± 0.3§)	4.5 ± 1.3† (66.8 ± 5.3†)	28.8 ± 1.9‡ (131.5 ± 4.8†)	41.2 ± 0.9‡ (112.2 ± 2.8†)
II	B10	5×10^7	4	7.3 ± 1.2	2.9 ± 0.4§ (2.2 ± 0.4§)	6.9 ± 1.9† (69.6 ± 3.7†)	31.7 ± 1.5§ (133.4 ± 3.6†)	42.5 ± 2.0† (103.4 ± 6.7§)
III	B10	1×10^7	4	0.8 ± 0.1	2.8 ± 0.3§ (2.2 ± 0.2†)	12.1 ± 1.1‡ (76.6 ± 2.4†)	30.7 ± 0.6§ (124.7 ± 3.3†)	42.3 ± 1.9† (97.0 ± 4.3§)
IV	B10	5×10^6	4	0.3 ± 0.0	2.4 ± 0.3† (1.9 ± 0.6§)	14.8 ± 1.7§ (88.3 ± 5.2‡)	32.0 ± 3.0§ (119.7 ± 1.1†)	40.7 ± 0.7† (96.9 ± 5.6§)

*The relative medianfluorescence intensity (MFI) was calculated by dividing the observed MFI of the peak of cells staining with mAbs for each analysis by the observed MFI of the same cell population from untreated B10 and multiplying the quotient by 100%.
†$P < 0.02$ compared with the untreated B10 mice.
‡$P < 0.05$ compared with the untreated B10 mice.
§N.S. compared with the untreated B10 mice.

ured by percentage MFI, was clearly augmented in all groups tested, although the augmentation of Ly-49D was lower in group IV mice compared with group I. Whereas, Ly-49D-positive cells were detected at the same frequency independent of the dose of injected allogeneic cells, as well as the percentage of D^d-positive cells found in spleen, which was consistent to the group 7 in Table 1. Exhibiting a similar trend, the augmentation of the expression of Ly-49G2, again in the percentage MFI, was observed in group I mice, but it was not seen in groups II, III and IV mice. Thus, it becomes clear that the expression of Ly-49 receptors on NK cells was also regulated by the dose of allogeneic H-2D^d-positive cells and the degree of downregulation or augmentation was regulated independently on NK cells.

The downregulated Ly-49A receptor inhibits NK cell activity functionally *in vitro*

We next analyzed the function of Ly-49 receptors *in vitro*. Four days after the injection of anti-Ly49A mAb, anti Ly-49D mAb, anti Ly-49G2 mAb or anti-NK 1.1 mAb, B10 mice were inoculated with 1×10^8 B10.D2 SC. Two days after the inoculation, SC were harvested from B10 recipients. The depletion of NK cells or Ly-49A, Ly-49D or Ly-49G2-positive NK cells was confirmed by FACSCalibur (data not shown) [22] at each examination as described in Materials and methods. NK cells from B10 mice which had been depleted Ly-49A-positive cells and inoculated with B10.D2 SC enhanced killing activity to H-2D^d target cells, compared with NK cells from B10 mice which were inoculated with B10.D2 SC alone (Fig. 3a). Although the expression of Ly-49A on NK cells was downregulated in untreated B10 mice which had been inoculated with B10.D2 SC alone, the killing activity of NK cells to H-2D^d target cells was sufficiently inhibited, compared with NK cells of SC from B10 mice injected with anti-Ly-49A mAb and inoculated with B10.D2 SC. Contrary to our expectation, NK cells from B10 mice which were depleted Ly-49D-positive cells and inoculated with B10.D2 SC showed more killing activity to H-2D^d-positive target cells compared with NK cells from B10 mice which were inoculated with B10.D2 SC alone (Fig. 3b). The killing activity of NK cells from B10 mice which were depleted Ly-49G2-positive cells and inoculated with B10.D2 SC was not significantly different from the killing activity of NK cells from B10 mice which were inoculated with B10.D2 SC alone (Fig. 3c).

The accelerated rejection of the H-2D^d-positive cells by the depletion of the Ly49-A *in vivo*

Finally, we examined the functional availability of Ly-49 receptors *in vivo*. Four days after the injection of anti-Ly-49A mAb, anti-Ly-49D mAb, anti-Ly-49G2 mAb or anti-NK1.1 mAb, B10 mice were inoculated with 2×10^7 or 1×10^7 B10.D2 SC. Two days after inoculation, the percentage and the number of H-2D^d-positive cells in the spleens of B10 mice were analyzed by FACSCalibur (Table 3). When B10 mice were inoculated with 2×10^7 B10.D2 SC after the depletion of Ly-49A-positive cells,

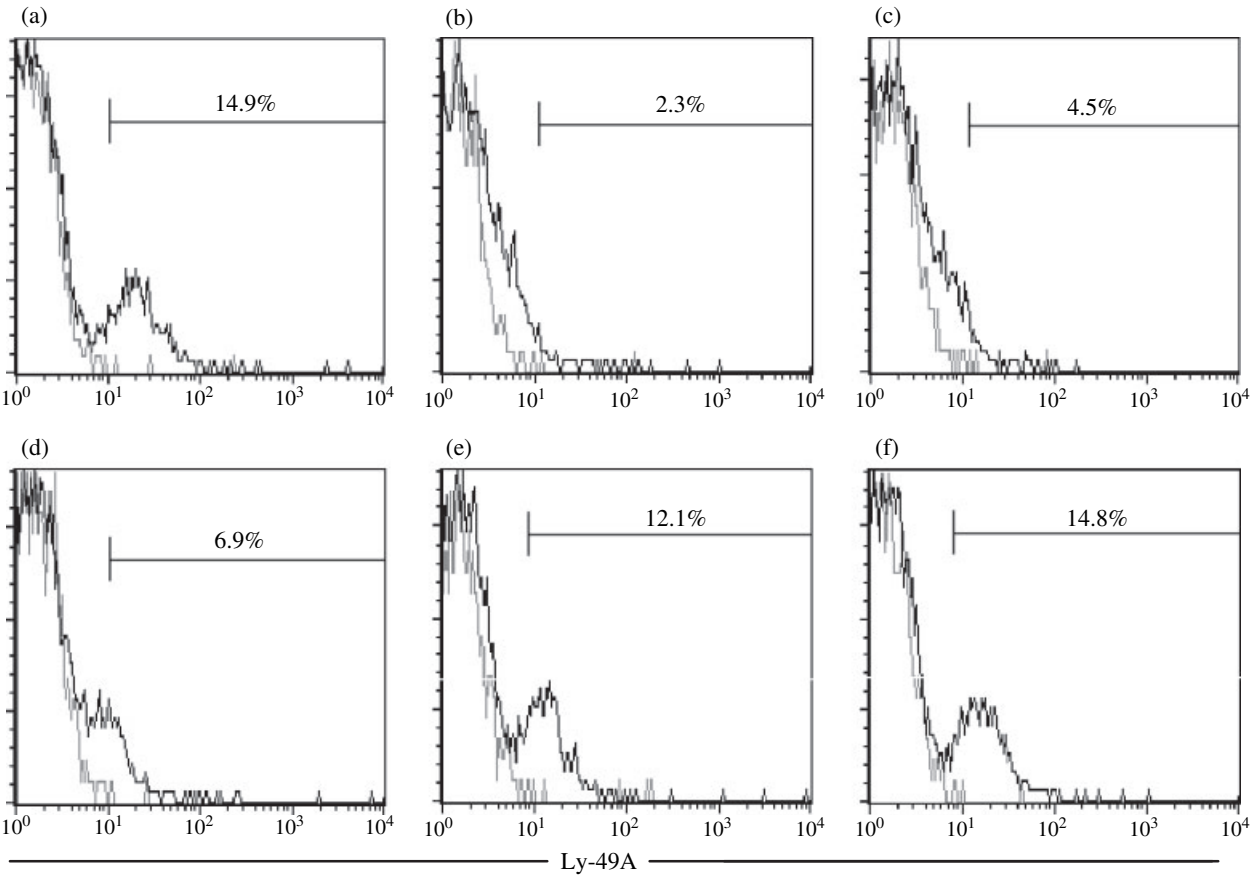


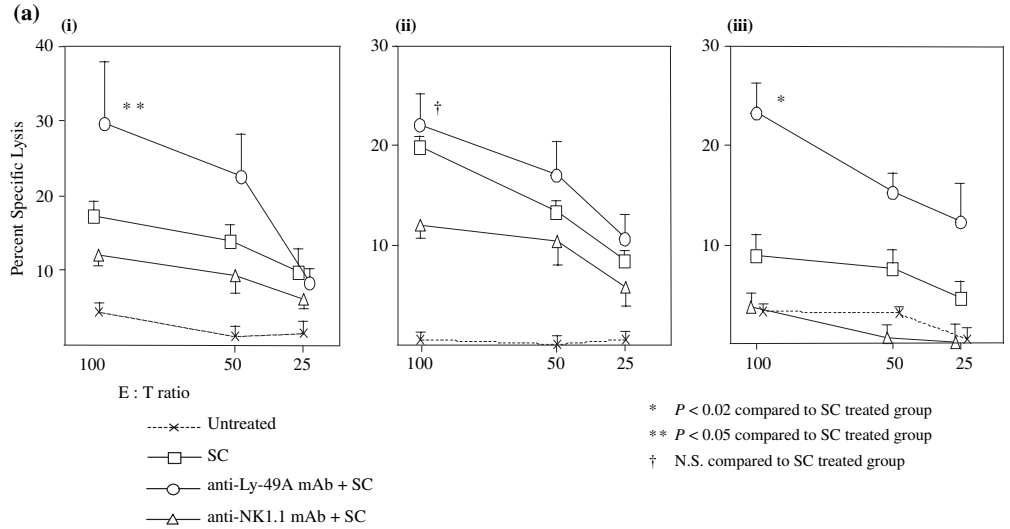
Figure 2 The downregulation of Ly-49A after the inoculation with different number of B10.D2 SC. Expressions of Ly-49A on NK cells in the spleen from (a) untreated B10 mice, (b) untreated B10.D2 mice and B10 mice which were inoculated with (c) 1×10^8, (d) 5×10^7, (e) 1×10^7 or (f) 5×10^6 B10.D2 SC. The splenocytes from B10 mice were tested 2 days after the inoculation and were analyzed by four-color fluorescence flow cytometory. The analysis gate was set on NK cells and histograms with the mean percentages of the Ly-49A$^+$ cells are shown. The data shown are from one of four experiments with representative results.

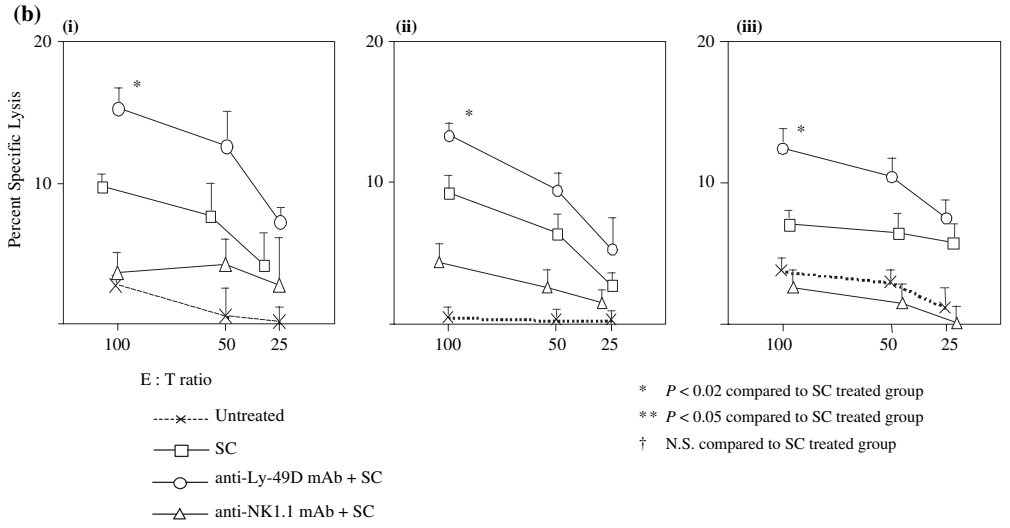
lower numbers of the inoculated B10.D2 SC were remained compared with non-depleted B10 mice (compared groups A to C). This was more evident when B10 mice were inoculated with a low does of 1×10^7 B10.D2 SC. Accelerated rejection was also observed after the depletion of Ly-49G2 with low dose of B10.D2 SC (compared group A to group E). When B10 mice were inoculated with B10.D2 SC after the depletion of Ly-49D-positive cells, higher percentage and higher number of D^d-positive cells were remained compared with non-depleted B10 mice (compared group A to groups D). Although the inhibitory Ly-49 receptors have been reported to function dominantly, a strong dominant inhibitory effect was not seen here, as the number of H-2D^d-positive cells were almost the same in percentage and in number in both the mice which were depleted for Ly-49D (group D) and for NK1.1-positive cells (group B).

Discussion

The receptor calibration theory hypothesizes that the calibration of inhibitory receptors against self-MHC enables NK cells to detect small alterations in MHC class I expression, and kills target cells which express low levels of MHC class I molecule on its surface such as tumor cells or virus-infected cells [17]. In this paper, we examined the regulation of Ly-49 receptors on NK cells *in vivo* during the process of the rejection of H-2D^d-positive allogeneic cells.

We showed that Ly-49A was downregulated quickly compared with the other Ly-49 receptors after the injection with H-2D^d-positive allogeneic cells. Furthermore, the level of downregulation was dose-dependent. It is known that Ly-49A interaction with H-2D^d-positive cells results in negative signaling [4], however, the present study provided the evidence that the downregulation of


(a)
(i)
(ii)
(iii)
Percent Specific Lysis
E : T ratio
Untreated
SC
anti-Ly-49A mAb + SC
anti-NK1.1 mAb + SC
* P < 0.02 compared to SC treated group
** P < 0.05 compared to SC treated group
† N.S. compared to SC treated group


(b)
(i)
(ii)
(iii)
Percent Specific Lysis
E : T ratio
Untreated
SC
anti-Ly-49D mAb + SC
anti-NK1.1 mAb + SC
* P < 0.02 compared to SC treated group
** P < 0.05 compared to SC treated group
† N.S. compared to SC treated group

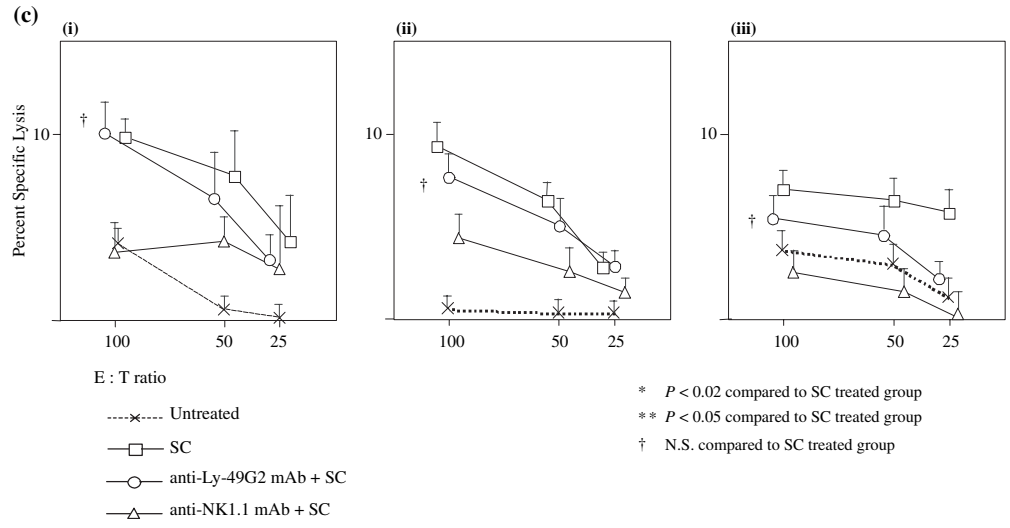

(c)
(i)
(ii)
(iii)
Percent Specific Lysis
E : T ratio
Untreated
SC
anti-Ly-49G2 mAb + SC
anti-NK1.1 mAb + SC
* P < 0.02 compared to SC treated group
** P < 0.05 compared to SC treated group
† N.S. compared to SC treated group

Table 3. The function of Ly-49A, Ly-49D and Ly-49G2 in B10 mice on the rejection of B10.D2 SC *in vivo*.

Protocol	Group	Mice	Preparation*	Number of Mice	Number of inoculated SC	D^{d+}cells (%)	Number of D^{d+} cells ($\times 10^6$/mouse)
High dose	A	B10	SC	5	2×10^7	2.3 ± 0.5	2.2 ± 0.1
	B	B10	anti-NK1.1 mAb + SC	5	2×10^7	3.2 ± 0.4‡	2.1 ± 0.1§
	C	B10	anti-Ly-49A mAb + SC	5	2×10^7	2.5 ± 0.5§	1.8 ± 0.3‡
	D	B10	anti-Ly-49D mAb + SC	5	2×10^7	3.6 ± 0.4†¶	2.4 ± 0.3§¶
	E	B10	anti-Ly-49G2 mAb + SC	5	2×10^7	2.4 ± 0.6§	1.9 ± 0.5‡
Low dose	A	B10	SC	5	1×10^7	1.5 ± 0.3	1.5 ± 0.4
	B	B10	anti-NK1.1 mAb + SC	5	1×10^7	2.3 ± 0.2†	1.7 ± 0.3‡
	C	B10	anti-Ly-49A mAb + SC	5	1×10^7	1.0 ± 0.2‡	0.7 ± 0.3†
	D	B10	anti-Ly-49D mAb + SC	5	1×10^7	2.3 ± 0.3†**	1.8 ± 0.2‡**
	E	B10	anti-Ly-49G2 mAb + SC	5	1×10^7	1.1 ± 0.2‡	0.9 ± 0.3‡

*Recipient mice were given mAb i.v. 4 days before the injection of B10.D2 SC to deplete NK cells or each subsets of NK cells. Depletion of NK cells and each subsets of NK cells were confirmed by flow cytometory before the injection of allogeneic cells.
†$P < 0.01$ compared with group A in the same dose protocol.
‡$P < 0.05$ compared with group A in the same dose protocol.
§N.S. compared with the untreated B10 mice.
¶$P < 0.05$ compared with group B in the same dose protocol.
**N.S. compared with group B in the same dose protocol.

Ly-49A expression on NK cells did not directly indicate NK tolerance.

The expression of Ly-49A on NK cells was downregulated and expressed only 4% of NK cells 2 days after the injection with B10.D2 SC. Contrary to our expectation, the cytotoxicity of the downregulated NK cells was definitely lower compared with Ly-49A-depleted NK cells. Furthermore, the depletion of Ly-49A resulted in the accelerated rejection of allogeneic SC in the *in vivo* model of B10.D2 SC injected B10 mice, relative to the control mice. Despite the downregulation of Ly-49A on NK cells exposed to $H\text{-}2D^d$ antigen, significant inhibitory signal remains allowing suppression of $H\text{-}2D^d$ antigen expressing targets. There is an inverse correlation between the levels of Ly-49A expression and the levels of the corresponding MHC-I ligand [23,24]. Ly-49A interacts with MHC-I in *cis* and *trans* [25], however, a causal relationship has not been identified. Our results imply two possibilities. Firstly, although the expression level of Ly-49A on NK cells is calibrated in response to available $H\text{-}2D^d$ antigen, its expression remains sufficient enough to preserve its inhibitory signaling function. Secondly, the downregulation was the process of delivering the negative signaling, and the lower killing activity was the consequence of the internalization of Ly-49A, which was found in internalization of CD3 [26]. As the relation between Ly-49A downregulation and its signaling is still unclear, further study is required to reveal the mechanism of this phenomenon.

The expression of activation receptor Ly-49D on NK cells of untreated B10.D2 mice was reduced, compare with that of untreated B10 mice (Table 1). The expression of Ly-49D on NK cells of B10 mice was augmented after the inoculation with B10.D2 SC in time dependently (Table 1) and also in dose dependently (Table 2). This augmentation of Ly-49D may be one of the mechanisms of the increased cytotoxicity of NK cells to $H\text{-}2D^d$-positive allogeneic cells. Although Ly-49D depleted B10 mice had retarded rejection of the inoculated B10.D2 SC *in vivo* (Table 3), cells isolated from similarly treated mice did not exhibit inhibited killing *in vitro* [8,22] (Fig. 3). It is possible that NK cells lyzed the targets using the injected anti-Ly-49D mAb 4E5 according to reversed ADCC. As the depletion of one subset of Ly-

Figure 3 The functional effects of Ly-49 receptors on NK cells in the rejection of D^d-positive target cells. (a) B10 mice which were treated with anti-Ly-49A mAb (open circle) or anti-NK1.1 mAb (open triangle) 4 days before inoculation or untreated B10 mice (open square) were inoculated with 1×10^8 B10.D2 SC. The splenocytes from B10 mice were tested 2 days after the inoculation with B10.D2 SC against (i) YAC-1, (ii) P815 and (iii) RL♂1 tumor cells. (b) B10 mice which were treated with anti-Ly-49D mAb (open circle) or anti-NK1.1 mAb (open triangle) 4 days before or untreated B10 mice (open square) were inoculated with 1×10^8 B10.D2 SC. The splenocytes from B10 mice were tested 2 days after the inoculation with B10.D2 SC against (i) YAC-1, (ii) P815 and (iii) RL♂1 tumor cells. (c) B10 mice which were treated with anti-Ly-49G2 mAb (open circle) or anti-NK1.1 mAb (open triangle) 4 days before or untreated B10 mice (open square) were inoculated with 1×10^8 B10.D2 SC. The splenocytes from B10 mice were tested 2 days after the inoculation with B10.D2 SC against (i) YAC-1, (ii) P815 and (iii) RL♂1 tumor cells. Data are presented as the percentage of specific cytotoxicity ± SD of triplicates. The data are a representative result from one of three experiments.

49 can change the profile of other Ly-49 expression [21], it is also possible that the depletion of Ly-49D-positive cells changed the profile of other receptors, which raised the NK activity by modulating the expression of other activation receptors, such as Ly-49H [7], or inhibitory receptors. We found that the duration of the modulation was different by each receptor, which was high-stepping in Ly-49A and tardy progress in Ly-49D and G2. Thus, the depletion of Ly-49D could reduce the activity of NK cells in initial phase, which retarded the rejection of allogeneic cells (Table 3, group D). However, the expression of other receptors could be modulated gradually and compensate the activity of NK cells, which could mediate strong killing activity in later (Fig. 3b).

The expression of Ly-49G2 is low in H-2^d or H-$2^{d/b}$ mice compared with H-2^b mice and downmodulation of Ly-49G2 has been observed in BALB/c into B6 mixed chimeras [20]. However, contrary to these findings, our studies have shown that the augmentation of expression of Ly-49G2 on NK cells following exposure to H-$2D^d$-positive allogeneic SC. Additionally, Ly-49G2 depleted B10 recipient mice had enhanced rejection of H-$2D^d$-positive allogeneic SC. Accelerated killing of target cells was not observed after the depletion of Ly-49G2 *in vitro* (Fig. 3c). The inconsistency from *in vivo* data could be due to the duration of other NK receptors to compensate the function. Thus, the depletion of Ly-49G2 might be effective in early phase, however, not effective in later phase. The inhibitory function of Ly-49A was clearly observed *in vivo* and *in vitro* (Fig. 3a and Table 3 group C), suggesting the dominant role of Ly-49A in the regulation of NK cells and other receptors could not compensate the function up to day 2. These findings indicate that in our model, the role of Ly-49G2 is at least as also important as the role of Ly-49A in mediating inhibitory signals on NK cells. The augmentation of Ly-49G2 in the allo response may serve to modulate NK activity and prevent excessive H-$2D^d$ target cell killing.

Our results strongly suggested the complexity of the NK cell receptors Ly-49A, Ly-49D and Ly-49G2 in the rejection of H-$2D^d$-positive allogeneic cells which were regulated in both duration of exposure and dose of antigen. The downregulation of Ly-49A, which was reported in a self- and allogeneic tolerance model, was also observed in our rejection model. However, the augmentation of Ly-49D and Ly-49G2 displayed a unique expression pattern in our allo-rejection model, which was not found in the allogeneic tolerance model. These findings provide new roles for 'receptor-calibration' of Ly-49 receptors in the regulation of NK cell activity.

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