Memory CD8 T lymphocytes express inhibitory MHC-specific Ly49 receptors

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Natural killer (NK) cells survey potential targets using an array of receptors specific for major histocompatibility complex class I molecules. In mice, members of the Ly49 receptor gene family are expressed on overlapping subsets of NK cells and on CD1-restricted NK1 T cells. Here we characterize a population of memory cytotoxic ($CD8^+$) T lymphocytes which also express inhibitory Ly49 family members. This cell population increases steadily with age; by 11 months, over one third of memory $CD8^+$ T cells express Ly49 molecules. These cells appear to express a normal TCR repertoire, and share several traits with previously activated T cells. Analysis of mutant mouse strains reveals that normal development of these cells depends upon the presence of the transporter associated with antigen presentation (TAP), classical class I molecules, and class II molecules. As a functional consequence of Ly49 expression, we demonstrate that T cell receptor-mediated activation of $CD8^+$ T cells is inhibited by Ly49 interactions with cognate class I molecules. We hypothesize that conventional memory $CD8^+$ T cells initiate Ly49 expression as a means of dampening an immune response and/or inhibiting T cell autoreactivity.

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1 Introduction

NK cells express receptors specific for MHC class I molecules, and it is believed that these receptors serve to prevent NK cells from attacking normal cells, while allowing them to attack infected or transformed cells in which class I molecules have been down-regulated [1]. Several classes of class I-specific receptors have been defined. Human NK cells often express one or more of the killer cell Ig-like receptors (KIR), which detect allelic specificities on MHC class Ia molecules [2]. In mice, most NK cells express one or more of the approximately nine or more members of the lectin-like homodimeric Ly49 family of receptors, which also exhibit allelic specificity for class Ia MHC molecules [3]. In addition, both human and mouse NK cells express heterodimeric CD94/NKG2 receptors specific for nonclassical class Ib molecules [4].

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The first two authors contributed equally to this work.

Abbreviations: TAP: Transporter associated with antigen presentation **KIR:** Killer cell Ig-like receptor **BrdU:** 5-Bromo-2'-deoxyuridine **poly(I:C):** Polyinosinic-polycytidylic acid

While expression of class I-specific inhibitory receptors is a common property of NK cells, evidence suggests that other cell types may less frequently express these receptors. In humans, CD94/NKG2 and KIR expression have been described on a subset of memory $CD8^+TCR\alpha\beta^+$ T cells, and infrequently on $CD4^+$ T cells [5]. Clones derived from KIR^+ cells appeared to be functionally inhibited, as they were impaired in their ability to secrete cytokines and to kill target cells bearing KIR-specific class I molecules [5]. It has been hypothesized that KIR expression by T cells may play a role in the inability to control some cancers or viral infections, as both HIV-specific and melanoma-specific $CD8^+$ T cell clones bearing functional KIR molecules have been isolated from patients.

There is limited comparable information in mice. A population of $CD4^+$ and double-negative $TCR\alpha\beta^+$ T cells with limited TCR diversity has been described which expresses the pan-NK cell marker NK1.1 and Ly49 family members. These "NK1 T cells" account for less than 1 % of splenocytes and cells in the lymph nodes, but make up about 15 % of mature thymocytes and about 25 % of T cells in the liver and bone marrow (for a review of NK1 T cells, see [6]). It has recently been demonstrated that the maturation and activation of NK1 T cells is restricted

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by the nonclassical class I molecule CD1 [6, 7]. Together, these data make it clear that NK1 T cells and conventional T cells are separate lineages with distinct functions. The role of inhibitory Ly49 receptors on these cells is currently unknown.

Although CD1-restricted NK1 T cells either express CD4 or are $CD4^-CD8^-$, it was recently noted that many of the peripheral $NK1.1^+$ T cells which express Ly49 molecules also express CD8 [8]. Additionally, Ly49 expression has been documented on $TCR\alpha\beta^+$ $CD8\alpha\alpha^+$ gut intraepithelial lymphocytes (IEL) which are thought to mature extrathymically [9]. In this report, we demonstrate that over 25 % of memory phenotype $CD8^+$ $TCR\alpha\beta$ T cells express Ly49 family members. We show that the Ly49-bearing $CD8^+$ T cell population diverges from NK1 T cells in both phenotype and developmental requirements, and we therefore propose that these cells represent a distinct stage in $CD8^+$ T cell activation, or possibly a novel T cell subset. Further, we show that TCR-mediated activation of these cells is inhibited by Ly49 interactions with class I molecules.

2 Results

2.1 Age-dependent expression of Ly49 receptors by $CD8^+CD4^-$ T cells

A significant population of peripheral $TCR\alpha\beta^+$ T cells was found to express Ly49 family members. As shown in Fig. 1 A, T cells in lymph nodes expressing Ly49G2 are almost entirely $CD4^-CD8^-$ or $CD4^-CD8^+$. Similar results were obtained using mAb specific for other inhibitory Ly49 receptors (Table 1, data not shown). Ly49-bearing $CD4^-CD8^-$ T cells include a unique population known as NK1 T cells [6]. However, the characteristics of $CD8^+$ T cells bearing Ly49 molecules have not been well documented.

Ly49 expression by $CD8^+$ T cells was evident in cells from lymph nodes, spleen, peripheral blood and liver (data not shown). In relatively young mice, a small percentage of $CD8^+$ T cells stained with anti-Ly49 mAb (Fig. 1 B, Table 1). Strikingly, the percentage of $Ly49^+CD8^+$ T cells expressing each Ly49 receptor increased steadily with age, resulting in a parallel increase in the total number of $Ly49A^+CD8^+$ T cells (Fig. 1 B, Table 1). By 11 months of age, between 4 % and 10 % of $CD8^+$ T cells in the spleen expressed each of the inhibitory Ly49 receptors for which mAb were available (Table 1). The percentages of $CD8^+$ T cells that expressed inhibitory Ly49 receptors were quite consistent among age-matched mice. We failed to observe appreciable expression of Ly49 receptors by peripheral $CD4^+$ T cells at any time point.

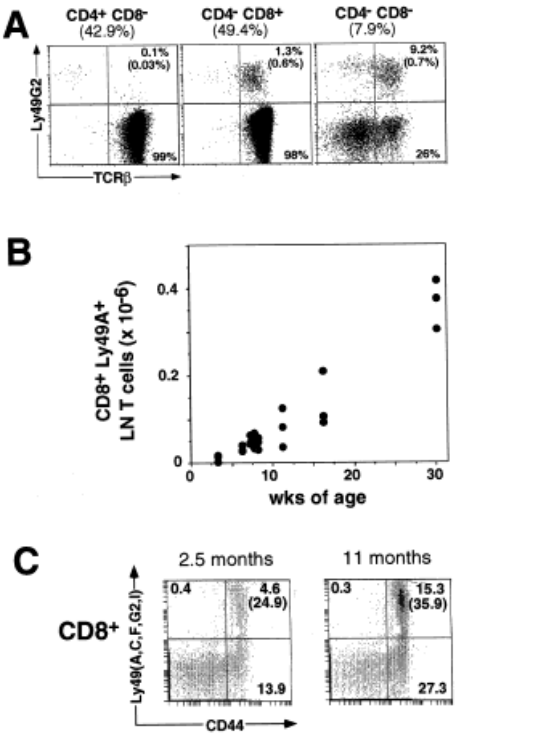

Fig. 1. Ly49 expression by T lymphocytes. (A) Ly49G2 expression on gated T cell subpopulations was determined using nylon wool-nonadherent lymph node cells from 16-week-old B6 mice. The percentages of total nylon wool-nonadherent cells are shown in parentheses. (B) The total number of lymph node $Ly49A^+CD8^+$ T cells was determined in B6 mice of different ages. Each point indicates an individual determination. (C) Nylon wool-nonadherent splenocytes from young (2.5 month) and old (11 month) B6 mice were stained with anti-CD8, anti-CD44, and a mixture of mAb specific for inhibitory Ly49 molecules (see Table 1); Gated $CD8^+$ cells are shown. The percentages of memory ($CD44^{hi}$) $CD8^+$ cells expressing at least one inhibitory Ly49 molecule are shown in parentheses.

To get a sense of the total percentage of $CD8^+$ T cells expressing inhibitory Ly49 receptors, a mixture of antibodies specific for Ly49A, C, I, F, and G2 was employed. These receptors comprise five of the seven characterized Ly49 receptors thought to be inhibitory. As shown in Fig. 1 C, approximately 4 % of $CD8^+$ T cells expressed one or more of these receptors in 8-week-old mice. By 11 months of age, $Ly49^+$ cells comprise approximately 14 % of $CD8^+$ T cells. The percentage of $CD8^+$ cells stained with the mixture of anti-Ly49 mAb was much lower than the sum of the cells expressing the individual Ly49 family members (Table 1) because, similar to the expression pattern on NK cells, different Ly49 family members were expressed on partially overlapping subsets of $CD8^+$ T cells (data not shown).

Table 1. Expression of Ly49 receptors by $CD8^+$ T cells and NK cells

		% of $CD8^+$ cells ± SD		% of $CD8^+$ $CD44^{hi}$ cells ± SD		% of $NK1.1^+$ cells ± SD
Ly49 mAb	Ly49	2.5 mo old	11 mo old	2.5 mo old	11 mo old	2.5 mo old
JR9	A	1.2 ± 0.0	4.1 ± 0.5	7.5 ± 0.4	10.9 ± 1.5	22.6 ± 2.6
5E6	C/I	1.0 ± 0.1	3.8 ± 0.2	5.0 ± 0.6	9.4 ± 1.2	46.5 ± 3.7
YLI	I	0.9 ± 0.1	3.6 ± 0.2	4.7 ± 0.2	9.1 ± 1.3	45.7 ± 2.5
HBF	F	3.5 ± 0.3	10.0 ± 1.4	17.7 ± 0.5	25.0 ± 1.0	10.9 ± 1.8
4D11	G2	2.2 ± 0.1	7.5 ± 0.9	11.0 ± 0.6	19.3 ± 1.3	42.4 ± 4.8
JR9+5E6+HBF+4D11	A/C/F/G2/I	4.3 ± 0.3	13.9 ± 1.8	22.7 ± 1.0	37.0 ± 1.9	76.1 ± 3.9
SED85	D	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	38.8 ± 6.9

Interestingly, essentially all of the Ly49-bearing $CD8^+$ T cells expressed high levels of CD44, indicative of a memory phenotype. Thus, the fraction of $CD8^+$ memory cells that express Ly49 receptors is considerable; in older mice, nearly 35 % of $CD44^+CD8^+$ T cells expressed one or more of the five inhibitory Ly49 receptors we tested (Fig. 1 C, Table 1). The comparison between younger and older mice revealed that the age-dependent increase in Ly49 expression by $CD8^+$ T cells is partly accounted for by an increased percentage of memory cells among $CD8^+$ T cells, and partly by an increased fraction of memory cells that express Ly49 receptors (Fig. 1 C, Table 1).

The pattern of Ly49 receptor expression by $CD8^+$ T cells differed in some respects from the pattern on NK cells. Using a novel anti-Ly49F mAb, we found that Ly49F was expressed more frequently than the other inhibitory receptors on $CD8^+$ T cells, despite being the Ly49 family member expressed at the lowest frequency on NK cells (Table 1). Furthermore, we and others failed to detect expression of the activating Ly49D receptor on T cells (Table 1, [8]). Ly49D is easily detected on approximately 40 % of NK cells in B6 mice (Table 1, [10]).

Phenotypic analysis revealed that some of the $Ly49^+CD8^+$ T cells also expressed other NK cell markers. For example, approximately one quarter of the cells expressed NK1.1, and nearly half expressed DX5 (Fig. 2). Essentially all $Ly49^+CD8^+$ T cells expressed high levels of CD44, CD122 (IL-2Rβ), Ly6C and Fas, and thus exhibit a memory phenotype (Fig. 2, data not shown). The cells did not appear to have been recently activated, as they did not express CD25 (IL-2Rα) or CD69, and were not large blasts as assessed by their forward and side light scatter characteristics (Fig. 2, data not shown). Interestingly, the $Ly49^+CD8^+$ T cells exhibited a broad range of CD8β expression, including some cells that failed to express significant levels of CD8β. The levels of CD8α and TCR surface expression by $Ly49^+CD8^+$ T cells were depressed by approximately 50 % compared to the level observed on $CD8^+$ T cells in general (Fig. 2).

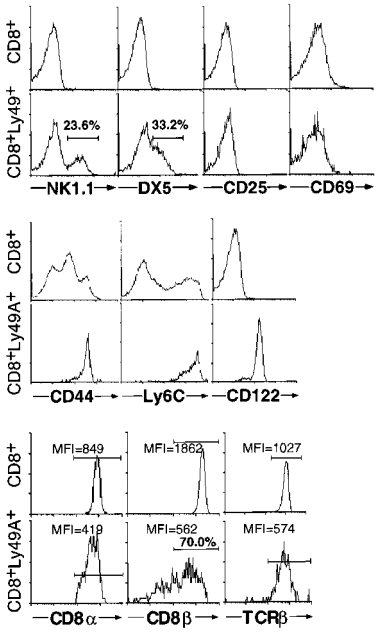

Fig. 2. Cell surface phenotype of $Ly49^+CD8^+$ T cells. The expression levels of cell surface molecules were examined on nylon wool-nonadherent lymph node cells from 12-week-old B6 mice. The expression on gated $CD8^+$ cells was compared to expression on gated $Ly49^+CD8^+$ cells expressing at least one inhibitory Ly49 molecule (see Table 1 for anti-Ly49 mAb mixture), or gated $Ly49A^+CD8^+$ cells. In some cases, mean fluorescence intensity (MFI) is shown.

2.2 Turnover of Ly49-bearing CD8+ T cells

To assess the proliferation rate of Ly49+ CD8+ T cells, 5-bromo-2'-deoxyuridine (BrdU) was used to label the DNA of dividing cells *in vivo*. We found that CD8+ T cells bearing Ly49A incorporated BrdU considerably more rapidly than Ly49A−CD8+ cells (Fig. 3 A), despite appearing to be small, non-blasting cells. In addition, a marked increase in Ly49+CD8+ T cell proliferation was induced by injection of synthetic double-stranded (ds)RNA polyinosinic-polycytidylic acid [poly(I:C)], with 43 % of these cells incorporating BrdU by day 3 (Fig. 3 B). The observed *in vivo* proliferation rate and poly(I:C) responsiveness of Ly49-bearing T cells are essentially identical to what has been documented for both CD8+ memory (CD44hi) cells [11, 12] and peripheral NK1 T cells (M.C.C. and D.H.R., unpublished results). Naive CD8+ T cells, on the other hand, proliferate slowly and respond poorly to poly(I:C) *in vivo* [11, 12].

2.3 TCR repertoire of Ly49-bearing CD8+ T cells

To assess whether the CD8+ T cells that express Ly49 receptors have an altered repertoire compared to other CD8+ T cells, we examined the Vβ repertoire of the cells.

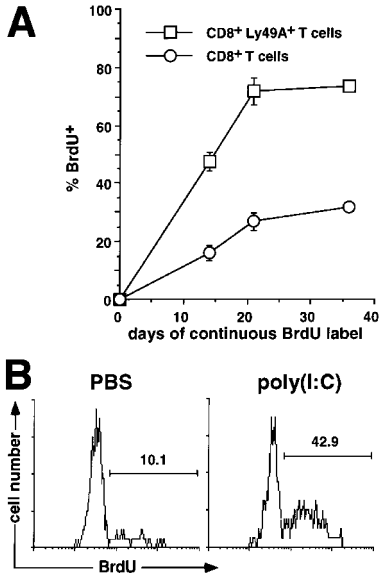

Fig. 3. Proliferation of Ly49+CD8+ T cells *in vivo*. (A) Eight-week-old B6 mice were administered BrdU continuously, and BrdU incorporation by gated CD8+ (○) and Ly49A+CD8+ (□) lymph node cells was determined by flow cytometry. (B) Eight-week-old B6 mice were injected with PBS (left) or poly(I:C) (right), followed by 3 days of BrdU treatment as above. Gated Ly49A+CD8+ lymph node cells are shown, with the percentage of BrdU-labeled cells indicated.

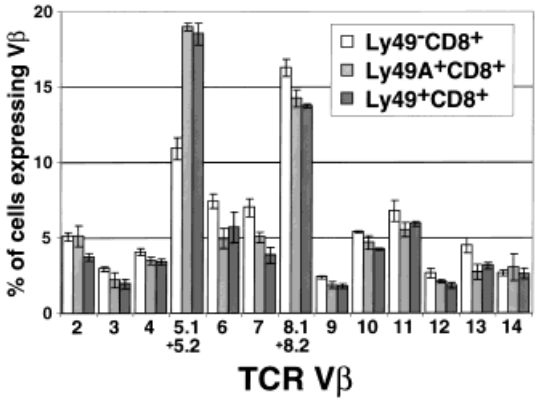

Fig. 4. TCR Vβ repertoire of Ly49+CD8+ T cells. Nylon wool-nonadherent splenocytes from 8-month-old B6 mice were stained with mAb specific for different Vβ, CD8, and different Ly49 molecules. The data are presented as the mean percentage (± SD, *n* = 4 individual mice) of the indicated gated population that are positive for the indicated TCR Vβ. "Ly49+" cells were stained using a mixture of anti-Ly49 mAb (see Table 1).

As shown in Fig. 4, all of the Vβ regions tested were well represented among Ly49+ CD8+ T cells. Furthermore, the frequencies of cells expressing each Vβ were in most cases very similar to the frequencies observed among Ly49−CD8+ T cells. Similar results were obtained using B10.D2, BALB/c and BALB.B mice (data not shown). Vβ5.1/5.2 was consistently expressed by a higher percentage of Ly49+CD8+ T cells compared to CD8+ cells in B6 mice (see Discussion). The Vβ repertoire data strongly suggest that the Ly49+CD8+ population is a polyclonal rather than oligoclonal population.

To investigate further the relationship between T cell receptor specificity and Ly49 expression, we examined T cell receptor transgenic mice expressing positively selecting MHC molecules. We observed little if any expression of Ly49 receptors by CD8+ T cells expressing high levels of the transgenic TCR in either H-Y or 2C TCR transgenic mice, while Ly49 expression can be detected on CD8+ cells bearing endogenous TCR (Fig. 5A, data not shown). As previously reported [13], essentially none of the clonotype-expressing CD8+ T cells expressed CD44 (data not shown), suggesting that they had not been stimulated with environmental antigens.

2.4 MHC dependence of Ly49 expression by CD8+ T cells

The role of MHC expression in the development of Ly49+CD8+ T cells was addressed using mutant mouse strains. Mice deficient for class I expression due to muta-

tion of the β2 microglobulin gene ($\beta2m^{-/-}$) exhibit a serve overall loss of $CD8^+$ T cells compared to wild-type mice, due to impaired positive selection of the cells ([14], Fig. 5 B). The number of $Ly49A^+CD8^+$ T cells was also sharply reduced in $\beta2m^{-/-}$ mice, but by a smaller factor, fivefold.

A similar effect was observed in mice deficient for class I expression due to a mutation in the transporter associated with antigen presentation (TAP-1) gene. TAP-1-deficient mice express somewhat higher class I levels than β2m-deficient mice [15]. Correspondingly, there was only a tenfold reduction in total $CD8^+$ T cells, and a fivefold reduction in $Ly49A^+CD8^+$ T cells. Likewise, in mice completely lacking class Ia expression ($K^{b-/-}D^{b-/-}$), there is an approximately tenfold reduction in $CD8^+$ T cells compared to normal B6 mice [16], and a fourfold reduction in total $Ly49A^+CD8^+$ T cells (Fig. 5 B). Together, these data suggest that the appearance of $Ly49^+CD8^+$ T cells is predominantly class I dependent, though perhaps not to the same extent as $Ly49^-$ T cells. Somewhat surprisingly, Ly49 expression by $CD8^+$ T cells was also affected by class II MHC expression. In $A\beta^{-/-}$ mice, which are devoid of classical class II molecules, there were approximately threefold fewer $Ly49^+CD8^+$ T cells compared to wild-type mice, despite having normal numbers of $CD8^+$ cells (Fig. 5 B).

To focus further on the role of class I expression in the appearance of $Ly49^+CD8^+$ T cells, we examined fetal liver irradiation chimeric mice. As shown in Fig. 5 C, normal numbers of $Ly49A^+CD8^+$ T cells were detected in control ($\beta2m^+ \rightarrow \beta2m^+$) fetal liver chimeras. As reported previously, almost no $CD8^+$ cells develop in ($\beta2m^{-/-} \rightarrow \beta2m^{-/-}$) chimeras, whereas ($\beta2m^+ \rightarrow \beta2m^{-/-}$) chimeras support the development of some $CD8^+$ T cells [approximately 10 % the number seen in control ($\beta2m^+ \rightarrow \beta2m^+$) chimeras] ([17], Fig. 5 C). Of these cells, the percentage expressing Ly49A was fivefold higher compared to the percentage in ($\beta2m^+ \rightarrow \beta2m^+$) control chimeras. Thus, the total number of $Ly49A^+CD8^+$ T cells in these chimeras was half that found in control chimeras, demonstrating that class I expression on hematopoietic cells leads to the development of some of these cells.

More informative were ($\beta2m^{-/-} \rightarrow \beta2m^+$) chimeras, in which class I expression is restricted to thymic epithelial cells and other non-hematopoietic cells. In these chimeras there was an approximately eightfold reduction in the percentage of $Ly49A^+CD8^+$ T cells compared to control chimeras, despite the presence of normal numbers of total $CD8^+$ T cells. Together, these data are consistent with the notion that Ly49 expression on T cells largely depends upon class I expression by hematopoietic cells, and that expression of class I by thymic epithelial cells and other non-hematopoietic cells is insufficient to support the appearance of normal numbers of these cells.

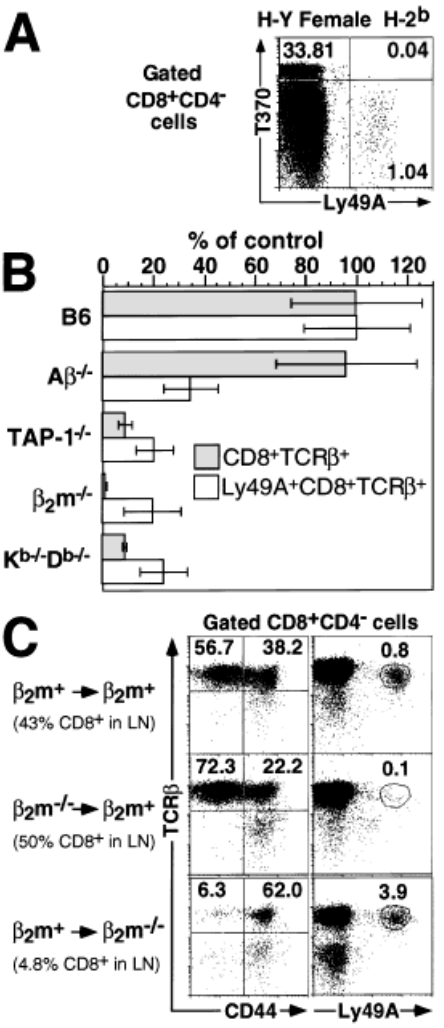

Fig. 5. Role of TCR and MHC molecules in the development of $Ly49^+CD8^+$ T cells. (A) Nylon wool-nonadherent lymph node cells from a female B6-H-Y TCR transgenic mouse were analyzed by flow cytometry: gated $CD8^+$ $CD4^-$ cells are shown. T3.70 mAb is specific for the H-Y TCRα chain. (B) Ly49 expression on $CD8^+$ cells in mice with defective MHC expression. Nylon wool-nonadherent splenocytes from 10-week-old B6 mice with homozygous mutations in the indicated genes were analyzed by flow cytometry. The data represent the relative mean percentage (± SD, $n \geq 3$ individual mice) in the spleen of the indicated cell population compared to wild-type B6 mice (defined as 100 %). When expressed as relative cell numbers, identical trends were observed. (C) Ly49A expression on $CD8^+$ T cells in fetal liver chimeras analyzed 8 weeks post-reconstitution. Gated $CD8^+CD4^-$ cells are shown; the percentages of nylon wool-nonadherent lymph node cells that are $CD8^+$ are shown in parentheses. Data in each case are representative of three to five individual chimeras.

2.5 Ly49-mediated inhibition of T cell activation

To assess the functional consequences of Ly49 expression on T cells, we tested whether polyclonal activation of Ly49A$^+$CD8$^+$ cells was inhibited in the presence of class I ligands recognized by Ly49A. Purified responder T cells from B6 mice were cultured with stimulator splenocytes depleted of T cells from B6 (H-2^b), B10.D2 (H-2^d congenic), or B6-β2m$^{-/-}$ mice. Anti-CD3 mAb was added at different concentrations to redirect stimulation of T cells via presentation by FcR$^+$ stimulator cells. Up-regulation of the early activation marker CD69 was measured on responder cell populations by flow cytometry. As shown in Fig. 6 A, Ly49A-bearing CD8 T cells readily up-regulated CD69 expression when stimulated by H-2^b or class I-negative stimulators in the presence of anti-CD3 mAb. However, activation was significantly inhibited when anti-CD3 mAb was presented by H-2^d stimulators, demonstrating that T cell activation can be blocked by H-2^d-Ly49A interactions. Importantly, CD69 up-regulation by memory and naive CD8 cells which did not express Ly49 molecules was unaffected by the H-2 haplotype of the stimulator cells (Fig. 6 B and C).

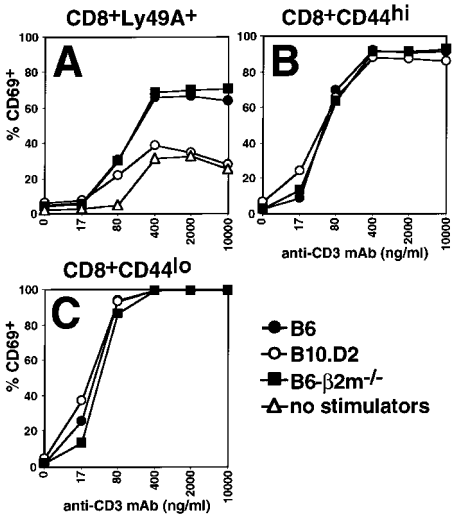

Fig. 6. Ly49 interactions with class I molecules inhibit CD3-mediated T cell activation. Purified T cell responders from 6-month-old B6 mice were cultured alone (Δ), or with T cell-depleted stimulator splenocytes from 3-month-old B6 (H-2^b, ●), B10.D2 (H-2^d, ○), or B6-β2m$^{-/-}$ (■) mice, in the presence of the indicated concentrations of anti-CD3 mAb. After 12 h, up-regulation of CD69 was determined by flow cytometry on (A) Ly49A$^+$CD8$^+$ cells, (B) Ly49$^-$CD8$^+$CD44^{hi} memory cells, or (C) Ly49$^-$CD8$^+$CD44^{lo} naive cells. A mixture of anti-Ly49 mAb (see Table 1) was used to allow gating of Ly49-negative cells. Data shown is representative of three separate experiments.

The level of Ly49A$^+$CD8$^+$ T cell activation induced by H-2^d stimulator cells matched that induced in the absence of stimulating cells (Fig. 6 A). This suggests that the activation observed in the presence of H-2^d stimulators can be attributed to plate-bound anti-CD3 mAb or mAb presentation by contaminating FcR$^+$ cells in the responder cell preparation.

3 Discussion

We have found that Ly49 molecules are expressed on a major subset of CD8$^+$ T cells bearing surface markers indicative of previous activation. Although these cells bear similarities to NK cells and NK1 T cells, our data suggest that Ly49 molecules are expressed on the surface of mature conventional CD8$^+$ T cells following antigenic stimulation. Ly49$^+$CD8$^+$ T cells differ from Ly49-expressing NK1 T cells in several respects. First, NK1 T cells express a restricted TCR repertoire, utilizing an invariant TCRα chain (Vα14-Jα281) and TCRβ chains containing Vβ2, 7, or 8 [6]. In contrast, we found that Ly49$^+$CD8$^+$ T cells express a diverse Vβ repertoire almost identical to conventional CD8$^+$ cells, with no overexpression of the canonical NK1 T cell Vβ (Fig. 4).

A second major difference between Ly49$^+$CD8$^+$ T cells and NK1 T cells is the role of class II, class Ia and class Ib molecules in their development. NK1 T cells are restricted by the nonclassical class I molecule CD1 in a TAP-independent manner. Therefore, NK1 T cells develop normally in mutant mice lacking TAP-1, classical class I, or class II (data not shown, [18–20]). Ly49$^+$CD8$^+$ T cells, on the other hand, are found in reduced numbers in all of these mice (Fig. 5 B). Similarly, the numbers of NK1.1$^+$Ly49$^+$CD8$^+$ T cells are comparably reduced in both *TAP-1*$^{-/-}$ and *K*$^{b-/-}$*D*$^{b-/-}$ mice (data not shown). Thus, neither Ly49$^+$CD8$^+$ T cells nor the fraction of these cells which express NK1.1 behave in the manner expected for CD1-dependent cells. Indeed, these cells are likely to correlate to the population of CD1-independent NK1.1$^+$ T cells described in the spleen [21].

Several features of Ly49 receptor expression on CD8$^+$ T cells suggest that a coordinated genetic program has been activated, similar to that in NK cells. First, all of the Ly49 family members for which mAb are available were expressed on CD8$^+$ T cells, with the notable exception of Ly49D (Table 1). Second, the expression of other NK markers such as NK1.1 and DX5 was predominantly ($>$ 80 %) restricted to Ly49$^+$CD8$^+$ T cells, with very little expression by Ly49$^-$CD8$^+$ cells (data not shown). Third, like NK cells, individual Ly49 family members were expressed on overlapping subsets of CD8$^+$ cells (data not shown).

We found that Ly49 expression on T cells and NK cells differs in some cases. First, the stimulatory Ly49D receptor was not detected on $CD8^+$ T cells (Table 1, [8]). This might imply that expression of activating NK cell receptors on T cells is disfavored, and that functional inhibition is the primary goal of Ly49 expression on these cells. Second, we generated a Ly49F-specific mAb and found that Ly49F was expressed on approximately 11 % of NK cells, but was expressed on approximately 70 % of $Ly49^+CD8^+$ T cells (Table 1). Ly49F is presumed to be an inhibitory receptor based upon the presence of an cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM), and we have recently reported weak interactions between Ly49F and $H\text{-}2^d$ products [22]. It is unclear why Ly49F is expressed on such a high percentage of $Ly49^+CD8^+$ T cells, but it raises the possibility that Ly49 receptor expression on T and NK cells is regulated differently.

Under what circumstances do $Ly49^+CD8^+$ T cells appear? Like conventional $CD8^+$ T cells, $Ly49^+CD8^+$ T cells required classical class I molecules for normal development (Fig. 5 B). It is interesting that the development of $Ly49^+CD8^+$ cells appears to be somewhat less affected by the absence of TAP-1, β2m, or K^b and D^b molecules than is the development of $CD8^+$ T cells in general. We conjecture that this difference is related to the fact that the vast majority of the remaining $CD8^+$ T cells in these mice bear a phenotype indicative of previous activation (IL-$2R\beta^+$, $CD44^{hi}$) (data not shown), which in normal B6 mice correlates with Ly49 expression on $CD8^+$ T cells. Indeed, the percentage of memory $CD8^+$ T cells that bear Ly49 receptors in these mutant mice is not elevated compared to normal B6 mice. Together, these findings suggest that $Ly49^+CD8^+$ T cells derive from, or develop in a manner similar to, memory $CD8^+$ CTL.

Under two conditions, reduced numbers of $Ly49^+CD8^+$ T cells were observed despite the normal development of other $CD8^+$ cells: in class II-deficient mice, and in ($\beta 2m^{-/-} \rightarrow \beta 2m^+$) chimeras (30 % and 12 % of normal, respectively; Fig. 5 B and C). Thus, as opposed to CTL, the development of normal numbers of $Ly49^+CD8^+$ T cells requires the expression of class II and the expression of class I on hematopoietic cells. These results might indicate that the appropriate expression of class I and class II are necessary for the positive selection of $Ly49^+CD8^+$ T cells in the thymus. However, we favor the hypothesis that Ly49 molecules are expressed on a CTL in the periphery only after it has been stimulated by class I on a BM-derived professional APC (which can provide co-stimulatory signals), and has received undefined signals from class II-dependent $CD4^+$ T cells. It is important to mention that approximately normal numbers of $CD44^{hi}CD8^+$ cells are found in both class II-deficient mice and ($\beta 2m^{-/-} \rightarrow \beta 2m^+$) chimeras (Fig. 5 C, data not shown); thus, the appearance of $Ly49^+CD8^+$ T cells does not always go hand in hand with the appearance of $CD44^{hi}$ memory CTL.

It is unclear why only some memory $CD8^+$ T cells express Ly49 molecules, and therefore the TCR specificities of $Ly49^+CD8^+$ T cells are of great interest. In the TCR transgenic mice we have examined, Ly49 molecules could not be detected on T cells expressing high levels of the transgenic TCR, but were expressed on a fraction of the $CD8^+$ T cells expressing endogenous TCRs (Fig. 5 A, data not shown). This may once again be an indication that prior stimulation is a prerequisite for Ly49 expression, or may reflect a requirement for particular TCR specificities not addressed by the available transgenes.

It is noteworthy that many human KIR-expressing $CD8^+$ cells appear to be monoclonally or oligoclonally expanded, and therefore express a heavily skewed TCR Vβ repertoire [23]. Although we did not observe evidence for significant Vβ skewing among $Ly49^+CD8^+$ T cells, the mice we examined were younger than 15 months old, the age at which oligoclonal expansions of $CD8^+$ T cells have been documented in some mice [24].

We consistently observed a slight overexpression of Vβ5 on $Ly49^+CD8^+$ T cells. Analysis of Vβ5 transgenic mice has shown that $V\beta5^+$ T cells in I-E-negative backgrounds (such as B6) undergo a gradual peripheral deletion, presumably due to poor presentation of the endogenous superantigen Mtv-9 Sag by I-A molecules [25]. It is therefore tempting to speculate that mature $V\beta5^+$ cells in B6 mice up-regulate Ly49 expression on "forbidden clones" as a result of chronic superantigenic stimulation. However, we have been unable to consistently draw similar correlations studying other Mtv superantigens and/or other reactive TCR Vβ in MHC-congenic mouse strains (data not shown).

What is the function of Ly49-expressing $CD8^+$ T cells? We have demonstrated that the TCR-mediated activation of these cells is inhibited as a consequence of Ly49 recognition of class I on antigen presenting cells. Therefore, if each of these cells express at least one self-specific Ly49 molecule (as is thought to be the case for NK cells), they will be retarded in their ability to respond to presented antigen. These data are in agreement with previous results showing that transgenic expression of Ly49A or Ly49G2 on $CD4^+$ and $CD8^+$ T cells inhibited the ability of these cells to proliferate in response to allogeneic stimulation and to respond to viral infection [22, 26, 27]. Similarly, in the human system, KIR have been documented to inhibit $CD8^+$ T cell functions [5].

Ly49$^+$CD8$^+$ T cells are also capable of killing target cells in a redirected lysis assay (data not shown). Cytotoxicity by sorted Ly49$^+$CD8$^+$ T cells cultured in IL-2 was found to be TCR dependent, in contrast to TCR-independent killing previously observed with Ly49$^+$CD3$^+$ lymphokine-activated killer cells (LAK) [8]. Somewhat surprisingly, we were unable to detect inhibition of CTL killing via naturally expressed Ly49 receptors (data not shown). Studies are currently underway to determine why we observe Ly49-mediated inhibition of T cell activation, but not downstream effector functions.

It is unknown at this point whether Ly49 expression on CD8$^+$ T cells is a transient state, or is a permanent expression pattern. It is possible that Ly49 molecules are up-regulated on responding CD8$^+$ T cell during some or all immune responses, serving as a common means for suppressing the response or as part of a genetic program leading to apoptosis of excess CD8$^+$ T cells. Conversely, perhaps Ly49 molecules are expressed on CD8$^+$ T cells undergoing chronic stimulation by self antigens, thereby inhibiting autoaggression. In either case, it is curious that a comparable population of Ly49$^+$ memory CD4$^+$ T cells does not exist. Taken together, these observations further the notion that the expression and function of inhibitory "NK cell" receptors is far more complex than originally thought, and may have far-ranging immunoregulatory functions on several different cells types.

4 Materials and methods

4.1 Mice

C57BL/6J (B6, H-2^b), B10.D2/nSnJ (B10.D2, H-2^d), BALB/cJ (BALB/c, H-2^d), B6-β2m$^{-/-}$ and B6-*TAP1*$^{-/-}$ mice were purchased from the Jackson Laboratory (Bar Harbor, ME). B6-$A\beta^{-/-}$ mice were purchased from Taconic (Germantown, NY). B6-H-Y TCR transgene [28] mice were backcrossed to B6 at least five times at the University of California, Berkeley. $K^{b-/-}$ $D^{b-/-}$ mice (backcrossed to B6) have been described [16].

4.2 Ly49D-, Ly49F-, and Ly49I-specific mAb

SED-85 (anti-Ly49D, IgG1) was generated by immunizing BALB/c mice four times i.p. with 5 μg of the soluble extracellular domain of Ly49D (B6 allele, produced by insect cells) in RIBI adjuvant (RIBI ImmunoChem Research, Inc., Hamilton, MT). HBF-719 (anti-Ly49F, IgG1), and YLI-90 (anti-Ly49I, IgG1) were generated by immunizing BALB/c mice i.p. four times with 5 × 10^6 CHO cells (CHO-K1, ADCC CCL-61) stably transfected with the B6 allele of Ly49F or Ly49I (respectively) in PBS. Splenocytes (10^8) from immunized mice were fused with 2 × 10^7 P3X63-Ag8.653 cells (ATCC TIB-9) using PEG 1500. HAT-resistant hybridoma supernatants were screened for specificity and lack of Ly49 cross-reactivity by flow cytometry using COS-7 cells transiently transfected with each of the B6 Ly49 (A–I) family members [22]. The mAb were purified from supernatants using protein G-Sepharose columns.

4.3 Antibody staining and flow cytometry

The following additional anti-Ly49 mAb were used: JR9–318 (anti-Ly49A, [9]), SW5E6 (anti-Ly49C/I, Pharmingen, San Diego, CA), and 4D11 (anti-Ly49G2, ATCC HB-240). Labeled mAb to the following molecules were purchased: CD4, CD8α, CD44, TCR Vβ4, 8, 10, 11, and 12 (Caltag Laboratories, Burlingame, CA); CD8β, TCRβ, CD25, CD122, Ly6C, CD69, TCR Vβ2, 3, 5, 6, 7, 13, and 14 (Pharmingen).

Nylon wool-nonadherent lymphocytes isolated from lymph nodes or spleen were preincubated 20 min with 2.4G2 (anti-FcγRII/III, ATCC HB-197) hybridoma supernatant. Staining with mAb was carried out in PBS containing 3 % FCS and 0.1 % NaN_3 for 30 min on ice. Flow cytometry analysis was performed on EPICS XL-MCL machines (Coulter Corp., Hialeah, FL).

4.4 BrdU labeling

BrdU (Sigma, St. Louis, MO) was added to drinking water at 0.8 mg/ml [11]. Lymph node cells were fixed, permeablized, DNase treated and stained with B44 mAb (anti-BrdU, Becton Dickinson, Sunnyvale, CA) as previously described [11]. To examine poly(I:C) (Sigma) induction of proliferation, mice were injected once i.p. with 100 μg poly(I:C) in PBS, followed by BrdU administered continuously (as above) for 3 days [12].

4.5 Fetal liver chimeras

Fetal (day 16) liver cells were dispersed through an 18-gauge needle, and 1.5 × 10^7 cells were injected intraorbitally into recipient mice which had been irradiated (980 rad) 2–4 h before. Recipient mice that could reject transplanted fetal liver cells were treated with 100 μg anti-NK1.1 mAb i.p. 24 h prior to irradiation.

4.6 *In vitro* stimulation assays

Responder T cells were prepared from nylon wool-nonadherent B6 splenocytes which were depleted of class II-positive cells using BP107 (anti-I-A, ATCC TIB-154) mAb ascites plus rabbit and guinea pig complement. Stimulator cells were prepared from splenocytes depleted of T cells using GK1.5 (anti-CD4, ATCC TIB-207) and AD4-15 (anti-CD8) mAb plus complement. The responder cell preparations contained less than 4 % B cells, and the stimulator cell preparations contained less than 3 % T cells. 1.5 × 10^6 stimulator cells were cultured with 0.5 × 10^6 responder cells and the indicated concentrations of 500A2 (anti-CD3ε, kindly provided by J. Allison) mAb for 12 h at 37 °C in 96-well

round-bottom plates which had been precoated with FCS to minimize mAb binding. Up-regulation of CD69 was determined by flow cytometry.

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