Expansion and Function of CD8$^+$ T Cells Expressing Ly49 Inhibitory Receptors Specific for MHC Class I Molecules[1]

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MHC class I-specific Ly49 inhibitory receptors regulate NK cell activation, thereby preventing autologous damage to normal cells. Ly49 receptors are also expressed on a subset of CD8$^+$ T cells whose origin and function remain unknown. We report here that, despite their phenotypic and cytolytic similarities, Ly49$^+$CD8$^+$ T cells and conventional Ly49$^-$CD44^{high} memory-phenotype CD8$^+$ T cells present strikingly distinct features. First, under steady state conditions Ly49$^+$CD8$^+$ T cells are poor cytokine producers (TNF-α and IFN-γ) upon TCR triggering. Second, Ly49$^+$CD8$^+$ T cells are not induced upon various settings of Ag immunization or microbial challenge. However, Ly49 can be induced on a fraction of self-specific CD8$^+$ T cells if CD4$^+$ T cells are present. Finally, the size of the Ly49$^+$CD8$^+$ T cell subset is selectively reduced in the absence of STAT1. These results indicate that Ly49 expression is associated with a differentiation program of cytolytic CD8$^+$ T cells triggered upon chronic antigenic exposure. They further suggest that the size of the Ly49$^+$CD8$^+$ T cell subset marks a history of CD8$^+$ T cell activation that might preferentially result from endogenous inducers of inflammation rather than from microbial infections. *The Journal of Immunology,* 2004, 173: 3773–3782.

Major histocompatibility complex class I molecules can be recognized by the TCR and CD8 molecules, thereby providing T cell stimulation. MHC class I molecules can also be recognized by a variety of cell surface receptors initially described on NK cells (1). These receptors include killer cell Ig-like receptors (KIR/CD158)[3] and leukocyte Ig-like receptors (leukocyte Ig-like receptors/Ig-like transcripts/CD85) in humans, Ly49 molecules in the mouse and CD94/NKG2 heterodimers in both species (2). Ly49 molecules in the mouse functionally correspond to human KIR. Both types of receptors belong to a multigenic and multiallelic family, recognize classical MHC class I molecules, and transduce inhibitory or activating signals depending whether they harbor an intracytoplasmic ITIM or associate with ITAM-bearing adaptors (3). Ly49 and KIR molecules also exhibit a very similar pattern of expression that includes NK cell and T cell subsets.

Ly49$^+$ T cells are mostly CD8$^+$ T cells with a memory phenotype (CD44^{high}, CD122$^+$, Ly6C$^+$), and the size of this subset increases with aging (4). It has been reported that engagement of Ly49 on T cells can inhibit T cell activation. However, as for inhibitory KIRs and LIR-1, the extent of the negative control induced by Ly49 is highly variable. Ly49 engagement can impair in vivo T cell responses in Ly49-transgenic mice (5–7). Yet, Ly49 engagement leads only to a limited down-regulation of CD69 induction in wild-type mice upon Ag exposure (4). In addition, inhibitory KIR and Ly49 molecules have also been reported to impair TCR-induced activation-induced cell death (8–12). Thus, it appears that inhibitory MHC class I receptors participate to the tuning of T cell response upon Ag encounter. In this study, we show that Ly49$^+$CD8$^+$ T cells can be considered as a subset harboring distinctive features compared with conventional memory-phenotype CD8 T cells that do not express Ly49 receptors. Moreover, we provide the first experimental evidence of in vivo Ag-specific expansion of Ly49$^+$CD8$^+$ T cells. Our results show that the expression of Ly49 molecules on CD8$^+$ T cells is associated with a history of persistent challenges and contributes to the functional heterogeneity of the CD8$^+$ T cell response.

Materials and Methods

Mice

Unless indicated, experiments were performed using 6- to 12-wk-old C57BL/6 mice. TCR-β-HY mice are transgenic for a TCR recognizing the Smcy3 peptide of the HY male Ag in the D^b context (13). MataHari are RAG-1$^{-/-}$ mice transgenic for a TCR recognizing the Uty peptide of the

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[3] Abbreviations used in this paper: KIR, killer cell Ig-like receptor; KARAP/DAP12, killer cell activating receptor-associated protein/DNAX activation polypeptide at 12 kDa; LCMV, lymphocytic choriomeningitis virus.

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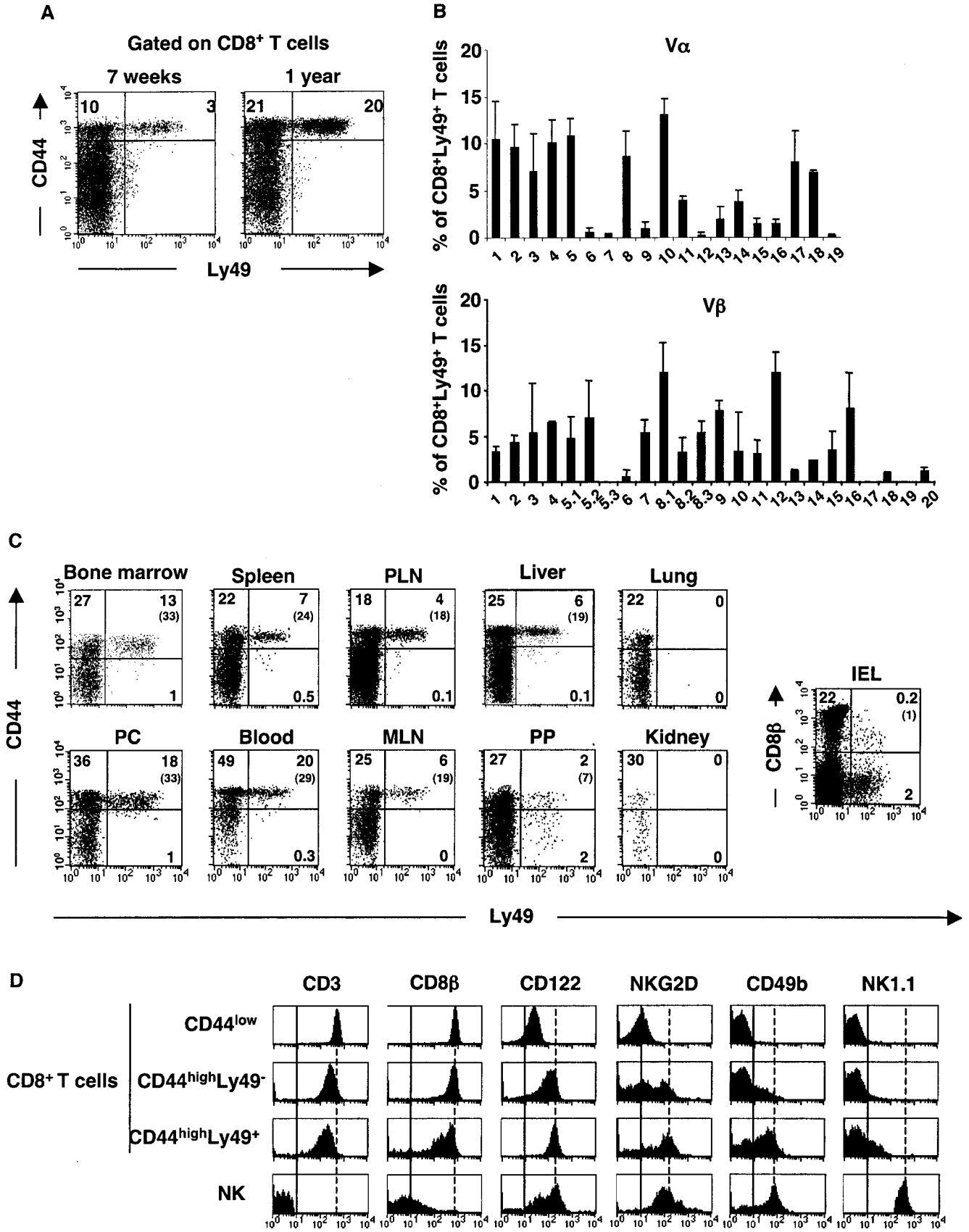

FIGURE 1. Phenotype and distribution of Ly49$^+$CD8$^+$ T cells. *A*, CD44 and Ly49 expression were analyzed on CD8α^+ splenocytes harvested from a 7-wk-old mouse (*left panel*) or a 1-year-old mouse (*right panel*). In these experiments, the cell surface expression of Ly49 molecules was assessed using a mixture of mAb recognizing inhibitory Ly49A, Ly49C/I, Ly49G, and Ly49F molecules. Numbers represent the percentages of cells in quadrants. The data presented are representative from a minimum of 10 experiments using mice at various ages. *B*, TCR repertoire of sorted Ly49$^+$CD8$^+$ T cells was assessed using the immunoscope technology described in *Materials and Methods*. Bars represent the mean percentage of Ly49$^+$CD8$^+$ T cells expressing each Vα or Vβ of two mice. SD are indicated. *C*, CD8α^+ cells harvested from spleen, blood, peripheral lymph nodes (PLN), (*Figure legend continues*)

HY male Ag in the D^b context (14). Marilyn are RAG-$2^{-/-}$ mice transgenic for a TCR recognizing the Dby peptide of the HY male Ag in the I-A^b context (15). IFN-$\gamma R^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). STAT$1^{-/-}$ mice were a generous gift from J. Durbin (The Ohio State University, Columbus, OH) and C. Biron (Brown University, Providence, RI) (16, 17). Experiments were conducted in accordance with institutional guidelines for animal care and use.

Immunofluorescence

Immunostainings were performed using the following Abs purchased from BD Biosciences (San Diego, CA): FITC-anti-CD25 (7D4, rat IgGM), FITC-anti-CD62L (MEL-14, rat IgG2a), PE or CyChrome-anti-CD44 (IM7.8.1, rat IgG2b), biotin-anti-CD3ϵ (145-2C11, hamster IgG1), biotin-anti-CD122 (TM-β1, rat IgG2b), biotin-anti-CD8β (53-5.8, rat IgG1), PerCP or allophycocyanin-anti-CD8α (53-6.7, rat IgG2a), PE-anti-NK1.1 (PK136, mouse IgG2a), PE-anti-CD49b (DX5, rat IgM). We also used unlabeled anti-NKG2D (MI6, rat IgG2a) (18) and allophycocyanin-anti-TCR-β (clone H57, hamster IgG; eBioscience, San Diego, CA). Unlabeled Abs were revealed using biotin-anti-rat (polyclonal mouse IgG; eBioscience). Biotinylated Abs were revealed using PerCP-streptavidin (BD Biosciences) or allophycocyanin-streptavidin (Caltag Laboratories, Burlingame, CA). The following FITC or biotin-anti-Ly49 mAb were used: anti-Ly49A (JR9.318, mouse IgG1, kindly obtained from J. Roland, Institut Pasteur, Paris, France), anti-Ly49A/D (12A8, rat IgG2a; BD Biosciences), anti-Ly49C/I (5E6, mouse IgG2a; BD Biosciences), anti-Ly49 C/I/F/H (clone 14B11, hamster IgG; eBioscience), anti-Ly49G (4D11, mouse IgG2a; BD Biosciences) and anti-Ly49F (HBF-719, mouse IgG1). Samples were analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

$Ly49^+CD8^+$ T cells were identified as $CD8\alpha^+$ lymphocytes that express any inhibitory Ly49 molecules detected by available mAb: anti-Ly49A, anti-Ly49C/I, anti-Ly49G and anti-Ly49F. In contrast to NK cells, no expression of activating Ly49 isoforms (Ly49D and Ly49H) was detected on T cells (data not shown), in agreement with previous reports (4, 19).

Immunoscope analysis

Quantification of PCR products was conducted as a competitive PCR strategy based on size-altered CD3ϵ cDNA, previously described by Ronet et al. (20), that allows for the determination of the number of CD3 copies contained in the samples. A volume of cDNA containing 10^4 copies of cDNA CD3ϵ was PCR amplified using each 19 Vα-specific or 24 Vβ-specific primers and a fluorescently labeled Cα- or Cβ-specific primer during 31 cycles (94°C, 30 s; 60°C, 30 s; 72°C, 30 s), thus remaining within the exponential phase of amplification. Products resulting of this PCR were analyzed on an automatic sequencer (Applied Biosystems, Foster City, CA). The size and the intensity of each band were recorded and then analyzed using Immunoscope software (Institut Pasteur).

Cell sorting

Cells from five to eight spleens were pooled and magnetically enriched in $CD8\alpha^+$ T cells by negative depletion using anti-CD4 (GK1.5, rat IgG2b), anti-B220 (RA3-6B2, rat IgG2a), and anti-rat-IgG-coated beads (Biomag; Metachem Diagnostics, Northampton, U.K.). Cells were then stained using a mix of FITC-anti-Ly49 (A-C/I-G-F), PE-anti-CD44, allophycocyanin-anti-CD8α. Samples were sorted using a FACSVantage SE cell sorter and FACSDiVa software (BD Biosciences).

Immunoblotting

Sorted cell subsets were lysed in a 1% Nonidet P40-containing solution for 30 min at 4°C. Lysates were then run in reducing condition on a 10–15% gradient polyacrylamide gel. After semidry transfer on an Immobilon-P membrane (Millipore, Bedford, MA) and incubation in saturating solution containing milk proteins, perforin, or killer cell activating receptor-associated protein/DNAX activation polypeptide at 12 kDa (KARAP/DAP12) were revealed using anti-perforin mAb (CB5.4, rat IgG2a; Alexis Biochemicals, Nottingham, U.K.) and anti-KARAP/DAP12 antiserum, respectively, followed by peroxidase-conjugated protein-A/G staining (Pierce, Rockford, IL). Peroxidase was revealed using ECL plus detection kit (Amersham Biosciences, Buckinghamshire, U.K.).

Cytotoxicity assay

^{51}Cr-labeled P815 cells (5×10^3) were incubated with serial dilutions of effector cells in the presence of anti-CD3 mAb (2C11) or isotypic control, in a standard 4 h cytotoxicity assay.

CFSE labeling

Cells were stained for 12 min at 37°C, in a 10 μM CFSE (Molecular Probes, Eugene, OR) containing medium (RPMI 1640) supplemented with 2% FCS.

In vitro cell stimulation

For cytokine production, 1×10^6 cells were stimulated for 6 h in anti-CD3ϵ mAb (2C11)-coated wells. Culture medium also contained brefeldin A (Sigma-Aldrich, St. Louis, MO) at 10 μg/ml. After surface staining, cells were permeabilized with PBS containing 1% saponin (Sigma-Aldrich) and 1% BSA (Invitrogen Life Technologies, Carlsbad, CA), and stained with PE-anti-IFN-γ (XMG1.2, rat IgG1; BD Biosciences) or PE-anti-TNF-α (MP6-XT22, rat IgG1; BD Biosciences). In some experiments, before TCR stimulation, cells were cultured for 3 days in the presence of 100 ng/ml recombinant mouse IL-15 (PeproTech, Rock Hill, NJ). In other experiments, naive cells were stimulated for 6 days in the presence of different combinations of following stimuli: anti-CD3ϵ mAb (2C11), anti-CD28 mAb (37.51, hamster IgG2; BD Biosciences) at 1 μg/ml, PMA (5 ng/ml; Sigma-Aldrich) with ionomycin (0.5 μg/ml; Calbiochem, San Diego, CA), IL-2 (Proleukin; Chiron, Emeryville, CA) at 1000 U/ml and IL-15 at 100 ng/ml.

Mice immunization

TCR-β-HY female mice were injected i.v. with 100 μl of PBS containing 100 μg of Smcy-3 peptide (KCSRNRQYL) and 50 μg of LPS (Sigma-Aldrich). Spleen, liver, and mesenteric lymph node cells were analyzed at day 7 upon immunization, after staining with PE-H-$2D^b$ multimers loaded with the Smcy-3 peptide, a mix of FITC-anti-Ly49 (A-C/I-G-F), CyChrome-anti-CD44, and allophycocyanin-anti-CD8α.

Adoptive transfers

Splenocytes were isolated from Marilyn and MataHari female mice. $CD4^+$ cells (5×10^5; Marilyn), $CD8^+$ cells (2×10^5; MataHari), or a mix of both were then injected i.v. in RAG-$1^{-/-}$ male mice.

Results

$Ly49^+CD8^+$ T cells are polyclonal cytolytic effectors

$Ly49^+CD8^+$ T cells are exclusively $CD44^{high}$ (Fig. 1*A*). The vast majority of them express $\alpha\beta$ TCRs (data not shown), although rare $Ly49^+CD8^+$ $\gamma\delta$ T cells have been described elsewhere (21). A complete analysis of $Ly49^+CD8^+$ $\alpha\beta$ TCR repertoire diversity was performed using immunoscope technology. As shown in Fig. 1*B*, the $Ly49^+CD8^+$ $\alpha\beta$ TCR repertoire is polyclonal and uses the various AV and BV rearrangements in the same proportions as previously reported for $CD8^+$ T cells from C57BL/6 mice and without biases in CDR3 size distribution (22). $Ly49^+CD8^+$ T cells represent a minor fraction of young adult mouse splenocytes (5.5% $\pm$ 1.7 of $CD8^+$ T cells, $n = 10$, 8- to 12-wk-old mice), but the size of the $Ly49^+CD8^+$ T cells subset increases with age (15% $\pm$ 4.4 of $CD8^+$ T cells, $n = 7$, 36- to 72-wk-old mice) (Fig. 1*A*).

mesenteric lymph nodes (MLN), liver, bone marrow, Peyer's patches (PP), peritoneal cavity (PC), lung, kidney were tested for CD44 and Ly49 expression or CD8β and Ly49 in the case of intraepithelial lymphocytes (IEL). Percentages indicated in quadrants are representative of two to six mice. Numbers in parentheses indicate the percentages of $Ly49^+CD8^+$ T cells within the $CD44^{high}CD8^+$ T cell compartment. *D*, Representative experiment showing expression of CD3, CD8β, CD122, NKG2D, CD49b, and NK1.1 on indicated splenocyte subsets harvested from a 10-mo-old female mouse. Different four color stainings were designed to allow analysis of the indicated molecule expression on naive $CD8^+$ T cells ($Ly49^-CD44^{low}CD8^+$), $Ly49^-CD44^{high}CD8^+$, $Ly49^+CD8^+$ T cells, and NK cells ($CD3^-NK1.1^+$). For each cell surface molecule, the dotted line indicates the maximum fluorescence intensity detected within the indicated lymphocyte subsets.

Table I. *Phenotypic features of Ly49$^-$ and Ly49$^+$CD8$^+$ T cells*[a]

	CD3	CD8β	CD122	NKG2D	CD49b	NK1.1
CD8$^+$CD44^{low}	427 ± 27	788 ± 55	23 ± 1	6 ± 1	3 ± 0	3 ± 0
CD8$^+$CD44^{high}Ly49$^-$	264 ± 16	596 ± 73	97 ± 20	38 ± 18	7 ± 1	11 ± 10
CD8$^+$CD44^{high}Ly49$^+$	183 ± 24	327 ± 46	179 ± 23	70 ± 15	25 ± 6	40 ± 18
NK cells	2 ± 0	13 ± 4	147 ± 16	161 ± 18	56 ± 2	277 ± 12

[a] Data represent the mean mean fluorescence intensity ± SD of cell surface CD3, CD8β, CD122, NKG2D, CD49b, and NK1.1 on indicated splenocyte subsets harvested from a 10-mo-old female mice. Cells were stained as in Fig. 1*D*. Data are collected from one representative experiment of three, performed with groups of two to four mice. The difference in mean fluorescence intensity for CD3, CD8β, CD122, NKG2D, and CD49b is statistically significant ($p < 0.05$) when CD8$^+$CD44^{high}Ly49$^-$ and CD8$^+$CD44^{high}Ly49$^+$ are compared. The difference in the percentage of NK1.1$^+$ cells is statistically significant ($p < 0.05$) when CD8$^+$CD44^{high}Ly49$^-$ and CD8$^+$CD44^{high}Ly49$^+$ are compared (data not shown).

Ly49$^+$CD8$^+$ T cells are found in spleen, in peripheral and mesenteric lymph nodes, in blood, in liver as well as in bone marrow (Fig. 1*C*). They are also present in the peritoneal cavity. Depending on the site, the size of the Ly49$^+$CD8$^+$ T cells subset varies from 18 to 33% of CD44^{high}CD8$^+$ T cells in 12- to 15-wk-old mice. Ly49$^+$CD8$^+$ are underrepresented in Peyer's patches, in small intestine intraepithelial lymphocytes, and virtually absent from nonlymphoid peripheral tissues such as lung and kidney (Fig. 1*C*). Therefore, in steady state conditions, Ly49$^+$CD8$^+$ mainly home within lymphoid organs in agreement with the expression of CD62L at their surface (data not shown).

The Ly49$^+$CD8$^+$ T cell surface phenotype was compared with that of naive CD8$^+$ T cells (CD44^{low}), Ly49$^-$ memory-phenotype CD8$^+$ T cells (CD44^{high}) and NK cells (Ly49$^+$CD8$^-$) using splenocytes from 45-wk-old mice. A modest decrease in the cell surface expression of CD3ϵ and CD8β contrasts with the modest increase in the cell surface expression of CD122 (the common β-chain for the IL-2 and the IL-15R complexes), the CD49b integrin (DX5), and NK1.1, when Ly49$^+$CD8$^+$ T cells were compared with naive CD8$^+$ T cells and Ly49$^-$CD44^{high}CD8$^+$ T cells (Fig. 1*D* and Table I). Along this line, a substantial fraction of freshly isolated Ly49$^+$CD8$^+$ T cells constitutively expresses NKG2D at the cell surface (46% ± 15 of Ly49$^+$CD8$^+$ T cells, $n = 9$, 12- to 30-wk-old mice) in contrast to the smaller percentage of NKG2D$^+$ cells within the Ly49$^-$CD44^{high}CD8$^+$ T cells (22 ± 8% of Ly49$^-$CD44^{high}CD8$^+$ T cells). On mouse CD8$^+$ T cells, NKG2D is induced upon TCR stimulation, but remains expressed at the T cell surface long (up to 8 mo) after antigenic stimulation has ceased, in contrast to CD25 and CD69 (18). The expression of NKG2D contrasts with the absence of CD69 and CD25 expression on Ly49$^+$CD8$^+$ T cells (data not shown), and suggests that in steady state conditions, Ly49$^+$CD8$^+$ T cells have returned to a more resting phenotype after having experienced previous stimulation. Thus, some cell surface phenotypic features distinguish Ly49$^+$CD8$^+$ T cells from Ly49$^-$CD44^{high}CD8$^+$ T cells, and position them with an intermediate phenotype between memory-phenotype CD8$^+$ T cells and NK cells (Fig. 1*D*). Yet, Ly49$^+$CD8$^+$ T cells, Ly49$^-$CD44^{high}CD8$^+$ T cells, and NK cells share the expression of cytolytic effector molecules, such as perforin (Fig. 2*A*), Fas ligand and granzyme B (data not shown). The cytolytic phenotype of both Ly49$^+$CD8$^+$ T cells and Ly49$^-$CD44^{high}CD8$^+$ T cells was confirmed by the ability of both types of sorted cells (but not of naive CD8$^+$ T cells) to effect anti-CD3 mAb-redirected cytolysis of FcR$^+$ P815 cells (Fig. 2*B*). Despite their resemblance to NK cells, freshly isolated Ly49$^+$CD8$^+$ T cells do not spontaneously kill classical NK cell targets such as YAC-1 and RMA/S (data not shown).

TCR-dependent triggering of Ly49$^+$CD8$^+$ T cells

The in vitro function of Ly49$^+$CD8$^+$ cells under steady state conditions was further investigated using cell proliferation as a first readout. CFSE-labeled splenocytes were stimulated using plate-bound anti-CD3 mAb or a mitogenic combination of PMA plus ionomycin. Both T cell subsets proliferated equally in response to anti-CD3 mAb or PMA plus ionomycin (Fig. 3*A*). This similarity in their proliferative response sharply contrasts with their difference in the ability to produce cytokines. Indeed, the percentage of Ly49$^+$CD8$^+$ T splenocytes that produce IFN-γ upon TCR engagement is greatly reduced as compared with that of Ly49$^-$CD44^{high}CD8$^+$ T cells (Fig. 3*B*, *upper panel*). As a control, only memory-phenotype CD8$^+$ T cells (CD44^{high}), but not naive CD8$^+$ T cells (CD44^{low}), produced IFN-γ over the 6 h stimulation period (Fig. 3*B*, *upper panel*). In contrast, both Ly49$^-$CD44^{high}CD8$^+$ T cells and naive CD8$^+$ T cells produce TNF-α in the same experimental settings. However, the fraction of Ly49$^+$CD8$^+$ T cells that produce TNF-α in response to this triggering is again markedly reduced (Fig. 3*B*, *lower panel*). The quantity of cytokine produced per Ly49$^+$CD8$^+$ T cell (mean fluorescence intensity of IFN-γ^+ cells and TNF-α^+ cells) is also reduced when compared with Ly49$^-$CD44^{high}CD8$^+$ T cells. In addition, the weak cytokine production induced by TCR stimulation of Ly49$^+$CD8$^+$ T cells was not reversed by addition of anti-MHC class I mAb (data not shown), suggesting that the potential engagement of inhibitory Ly49 molecules (e.g., Ly49C/I) by H-2^b molecules was not involved in this defect. No production of IL-2, IL-4, IL-5, IL-10, TGF-β by Ly49$^+$CD8$^+$ T cells was detected under these conditions (data not shown). Therefore, Ly49$^+$CD8$^+$ T cells appear to be defective for Tc1 response upon TCR stimulation, in contrast to the Ly49$^-$CD44^{high}CD8$^+$ T cell subset.

TCR-dependent expansion of Ly49$^+$CD8$^+$ T cells

The mechanisms that lead to the induction of Ly49$^+$CD8$^+$ T cells are still unknown. The selective expression of Ly49 on memory-phenotype CD8$^+$ T cells guided us to analyze the conditions that could lead to the expansion of Ly49$^+$CD8$^+$ T cells. First, the transcription factors, T-bet and Eomesodermin, have been recently shown to participate to CD8$^+$ T cell, NK cell, and NKT cell effector differentiation (23–25). Thus, we directly investigated whether the ectopic expression of these transcription factors was sufficient to induce Ly49 expression on the surface of CD8$^+$ T cells. However, naive T cells isolated from lymphocytic choriomeningitis virus (LCMV)-specific TCR transgenic mice (P14) and retrovirally transduced with T-bet and Eomesodermin constructs (alone or in combination) failed to express cell surface Ly49 upon in vitro culture with cognate peptide and IL-2 (data not shown). Second, we attempted to dissect the circumstances of TCR stimulation that could lead to the induction of Ly49 expression on the T cell surface in vivo. To increase the frequency of Ag-specific cells in a polyclonal T cell population, we first used transgenic mice expressing a TCR-β chain that reacts with the Smcy3 peptide from the male HY protein presented by D^b (TCR-β HY mice). Despite the in vivo expansion of Ag-specific CD8$^+$ T cells upon

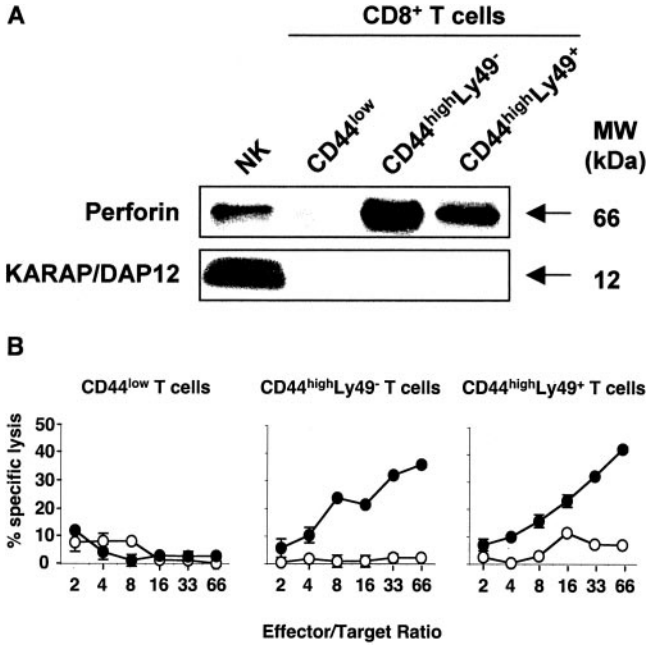

FIGURE 2. Cytolytic potential of Ly49$^+$CD8$^+$ T cells. *A*, Perforin and KARAP expression were analyzed by immunoblotting of cell lysates derived from indicated sorted cell subsets. *B*, The cytotoxicity of naive CD8$^+$ T cells (Ly49$^-$CD44^{low}CD8$^+$), Ly49$^-$CD44^{high}CD8$^+$, and Ly49$^+$CD8$^+$ T cells against P815 mastocytoma was monitored using a redirected killing assay. Freshly sorted cell subsets were incubated for 4 h with ^{51}Cr-labeled P815 cells at indicated E:T ratios in the presence of anti-CD3 mAb (●) or an isotypic control (○) at 10 μg/ml. Results are representative from three independent experiments.

Smcy3 peptide challenge (assessed by the use of Smcy3/D^b multimers), no significant induction of Ly49 molecules on these HY-specific T cells was observed (Fig. 4*A*). Repeated challenges with the Smcy3 peptide also failed to induce HY-specific Ly49$^+$CD8$^+$ T cells (data not shown). Similarly, *Listeria monocytogenes* challenge of CB6 (BALB/c × C57BL/6) wild-type mice in vivo led to the expansion of *L. monocytogenes*-specific CD8$^+$ T cells restricted by H2-M3 or K^d (26), but no Ly49 molecule was detected on H2-M3- or K^d-specific T cells upon primary or secondary stimulation in vivo using H2-M3 or K^d multimers (data not shown). Finally, the expansion of Ly49$^+$CD8$^+$ T cells during the course of CD8$^+$ T cell "homeostatic" proliferation was also investigated using the adoptive transfer of sorted CD44^{low}CD8$^+$ T cells from P14 transgenic mice into RAG-1$^{-/-}$ lymphopenic hosts (27). Despite the proliferation of P14 CD8$^+$ T cells when transplanted into lymphopenic recipient mice, no cell surface expression of Ly49 was detected on these cells (data not shown). Consistent with previous results (28), the apparent discrepancy between the selective expression of Ly49 on memory-phenotype T cells and the lack of Ly49 induction of Ag-specific T cells using three distinct experimental models of antigenic stimulation thus shows that the induction of Ly49 expression on the surface of Ag-experienced CD8$^+$ T cells is a rare event that likely requires a complex series of T cell triggering.

Along this line, the involvement of inhibitory Ly49 molecules in NK cell tolerance to self has led to the hypothesis that these molecules might subserve the same function in T cells (5). We thus asked whether the expression of Ly49 was the hallmark of self-reactive CD8$^+$ T cells, using the HY-specific model. Yet, in TCR-β HY male mice, most of the potentially self-reactive CD8$^+$ T cells (Smcy3/D^{b+}CD8$^+$ T cells) do not express inhibitory Ly49 molecules (Fig. 4*A*). However, thymic selection leads to the elimination of Smcy3/D^b multimer^{high} CD8$^+$ T cells in male mice (13), leading to the possibility that the Smcy3/D^b multimer^{low} CD8$^+$ T cells present in male mice are not bona-fide efficient autoreactive T cells. To avoid this caveat, we performed adoptive transfer experiments of HY-specific mature T cells isolated from a female

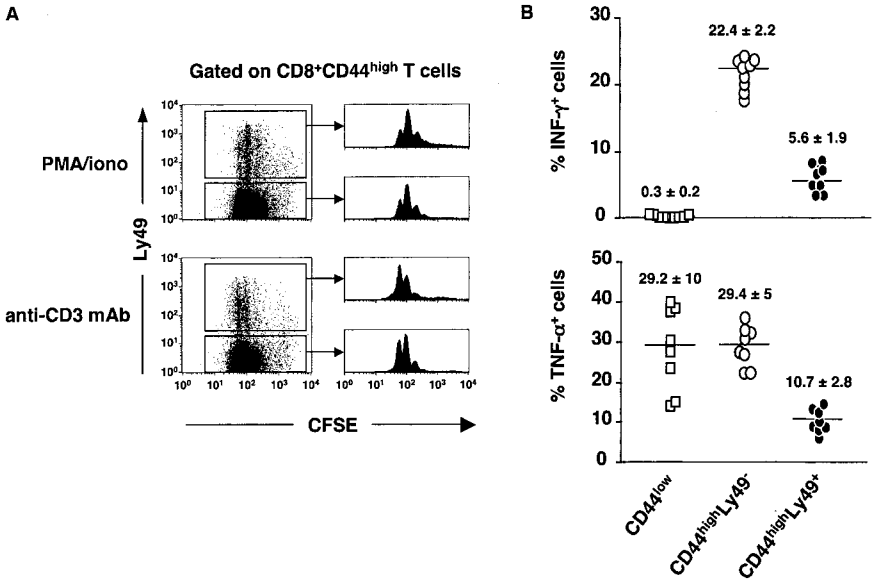

FIGURE 3. In vitro TCR triggering of Ly49$^+$CD8$^+$ T cells. *A*, Proliferation of splenocytes was assessed using CFSE. Splenocytes were cultured for 3 days under indicated stimuli. Anti-CD3 mAb was immobilized at 10 μg/ml. PMA and ionomycin were used at 5 ng/ml and 0.5 μg/ml, respectively. Before FACS analysis, cells were stained with a mix of anti-Ly49, anti-CD44, and anti-CD8α mAb. Dot plots are gated on CD44^{high}CD8α^+ T cells. Histograms are gated on Ly49$^+$CD8$^+$ T cells or Ly49$^-$CD44^{high}CD8$^+$ T cells. Results are representative from three independent experiments conducted with groups of two or more mice. *B*, Splenocytes from C57BL/6 mice were stimulated for 6 h with plate-bound anti-CD3 mAb in the presence of brefeldin A. Productions of IFN-γ (*upper panel*) and TNF-α (*lower panel*) were then assessed by cytometry on naive CD44^{low}CD8$^+$ cells (□), Ly49$^-$CD44^{high}CD8$^+$ T cells (○), and Ly49$^+$CD8$^+$ T cells (●). After cell surface staining for CD8α^+, CD44, and Ly49 molecules, cells were fixed, permeabilized, and intracellularly stained with anti-IFN-γ or anti-TNF-α. Mean ± SD of secreting cell percentages are indicated.

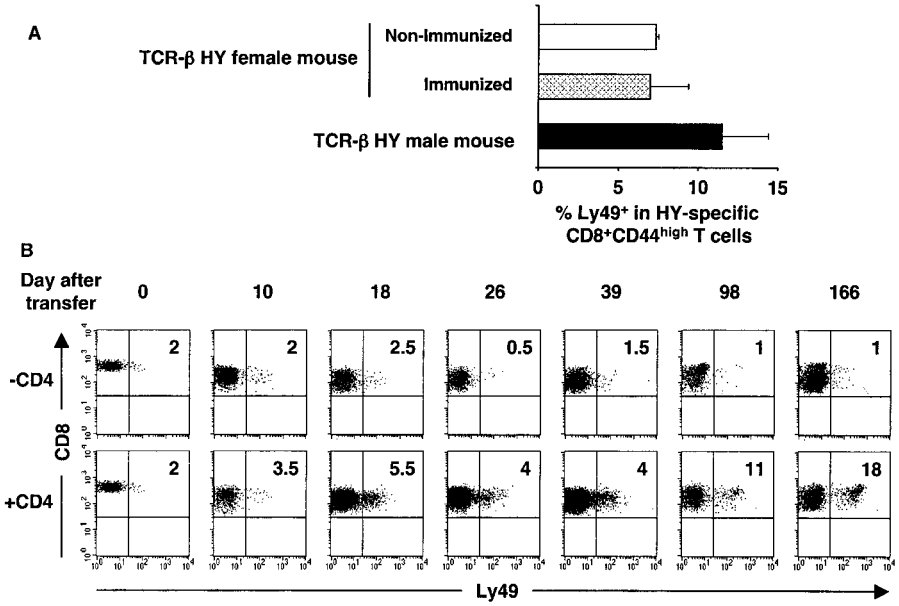

FIGURE 4. In vivo induction of Ly49$^+$ CD8$^+$ T cells. *A*, TCR-β-HY female mice were immunized with Smcy3 peptide. Ly49 expression on specific CD8α^+CD44^{high} splenocytes (Smcy3/D^b multimer$^+$) was assessed by cytometry before (□) and 7 days after peptide challenge (▨). Splenocytes harvested from TCR-β-HY male mice were stained with Smcy3/D^b multimer, anti-CD8α, anti-CD44, and a mix of anti-Ly49 (■). Results represent the mean percentages ± SD of splenic Ly49$^+$CD8$^+$ T cells in groups of three or more mice. *B*, Splenocytes from MataHari female mice (CD8$^+$ T cells) were i.v. transferred to RAG-1$^{-/-}$ male mice in the presence or absence of splenocytes from Marilyn female mice (CD4$^+$ T cells). Ly49 acquisition on CD8$^+$ T cells was assessed by flow cytometry on blood lymphocytes isolated at the indicated time points. Results are representative from three independent experiments conducted with groups of three or more mice.

mouse into a male recipient. In these experiments, we use HY-specific CD8$^+$ T cells isolated from female Uty/D^b-specific TCR transgenic mice (MataHari mice) as well as HY-specific CD4$^+$ T cells isolated from female Dby/I-A^b-specific TCR transgenic mice (Marilyn mice) (14, 15). These cells were adoptively transferred alone or in combination in lymphopenic RAG-1$^{-/-}$ male recipient mice. Starting from undetectable levels of Ly49$^+$CD8$^+$ T cells, a gradual and slow expansion of Ly49$^+$CD8$^+$ T cells was observed over time, but only in the presence of CD4$^+$ T cells (Fig. 4*B*). Interestingly, the expression of Ly49 is only detected on a fraction of Ag-specific CD8$^+$ T cells, despite their TCR clonality. As a control, no CD8$^+$ HY-specific T cells can be recovered when injected in female mice. Thus, these data document an Ag-specific expansion of Ly49$^+$CD8$^+$ T cells upon persistent antigenic challenge and in the presence of CD4$^+$ T cells.

Bystander activation of Ly49$^+$CD8$^+$ T cells

In addition to TCR-dependent T cell activation, a so-called "bystander" T cell proliferation has been reported upon mouse treatment with TCR-independent stimuli, such as poly(I:C) and LPS, that leads to IL-15 production (29). Ly49$^+$CD8$^+$ T cells readily proliferate upon IL-15 treatment in vitro (Fig. 5*A*), consistent with a recent report (30). In contrast, a much lower fraction of CD8$^+$CD44^{high}Ly49$^-$ T cells entered into proliferation, and naive CD8$^+$CD44^{low} T cells appear insensitive to IL-15 stimulation (Fig. 5*A*). Interestingly, the response of Ly49$^+$CD8$^+$ T cells to IL-15 has other functional consequences. Indeed, the Ly49$^+$CD8$^+$ T cell defect in cytokine production was reversed by treatment with IL-15 before TCR engagement. Upon 3 days of IL-15 treatment, the fraction of cells that produce IFN-γ and TNF-α upon anti-CD3 mAb stimulation was greatly increased in the Ly49$^+$CD8$^+$ T cell subset and became only slightly different from that of Ly49$^-$CD44^{high}CD8$^+$ T cells (Fig. 5*B*).

The possibility that IL-15 does induce the cell surface expression of Ly49 on T cells was then investigated. Sorted CD8$^+$CD44^{low} T cells (Ly49$^-$) were labeled with CFSE and cultured in the presence of IL-15, PMA plus ionomycin, plate-bound anti-CD3 plus anti-CD28 mAb stimulation alone or in combination with IL-2 and/or IL-15, and assayed for their cell surface expression of Ly49 molecules. None of these in vitro treatments induced Ly49 at the T cell surface despite the vigorous proliferation and the up-regulation of CD44 observed upon anti-CD3 plus anti-CD28 mAb in the presence or absence of cytokines (Fig. 6). Thus, IL-15 preferentially promotes the expansion of the Ly49$^+$CD8$^+$ T cell subset without inducing Ly49 molecules at the T cell surface.

It has been proposed that bystander proliferation of T cells depends upon the type I IFN pathway that initiates in turn the production of IL-15 (31). STAT-1 is an essential mediator of the type I IFN receptor (IFNAR) signaling pathway (16, 32). We found that the size of the Ly49$^+$CD8$^+$ T cell subset is selectively reduced within the CD44^{high}CD8$^+$ T cell compartment in STAT1$^{-/-}$ mice (Fig. 7*A*, *upper panel*). The repertoire of the Ly49$^+$CD8$^+$ T cell remains unchanged in STAT1$^{-/-}$ mice (Fig. 7*B*, *upper panel*), indicating a general defect in induction and/or survival of Ly49$^+$CD8$^+$ T cells in the absence of STAT-1. In contrast, no alteration of NK cell number, nor Ly49 expression on NK cells, was observed in STAT1$^{-/-}$ mice (Fig. 7, *lower panels*). The unavailability of IFNAR$^{-/-}$ mice in a pure C57BL/6 background prevented us from directly addressing the role of type I IFN in controlling the size of the Ly49$^+$CD8$^+$ T cell subset. In addition to its role in IFNAR signaling, STAT-1 is also involved downstream of IFN-γ receptor (IFNGR) and IL-27 receptor engagement (33, 34). Yet, no alteration of the Ly49$^+$CD8$^+$ T cell subset was observed in IFNGR$^{-/-}$ mice (Fig. 7*A*, *upper panel*) or in IL-27$^{-/-}$ mice (EBI3$^{-/-}$ mice (35), data not shown), supporting a critical role of the type I IFN pathway in regulating the size of the Ly49$^+$CD8$^+$ T cell subset in vivo.

Discussion

Ag encounter gives rise to distinct subsets of Ag-experienced T cells that include effector, central memory, and effector memory cells (36–40). Although the precise lineage relationship between these subsets is still a matter of debate, it is clear that T cell activation generates phenotypic and functional heterogeneity. We report here a characterization of the subset of CD8$^+$ T cells that express inhibitory Ly49 receptors for MHC class I molecules. Ly49$^+$CD8$^+$ T cells share several features with Ly49$^-$ memory-phenotype T cells, such as the high cell surface density of CD44 and Ly6C, the intracytoplasmic expression of perforin and the TCR-dependent cytolytic potential. However, Ly49$^+$CD8$^+$ T cells are distinguishable from conventional memory-phenotype Ly49$^-$CD44^{high}CD8$^+$ T cells in several ways, providing clues as to the heterogeneity of the intraclonal CD8$^+$ T cell response

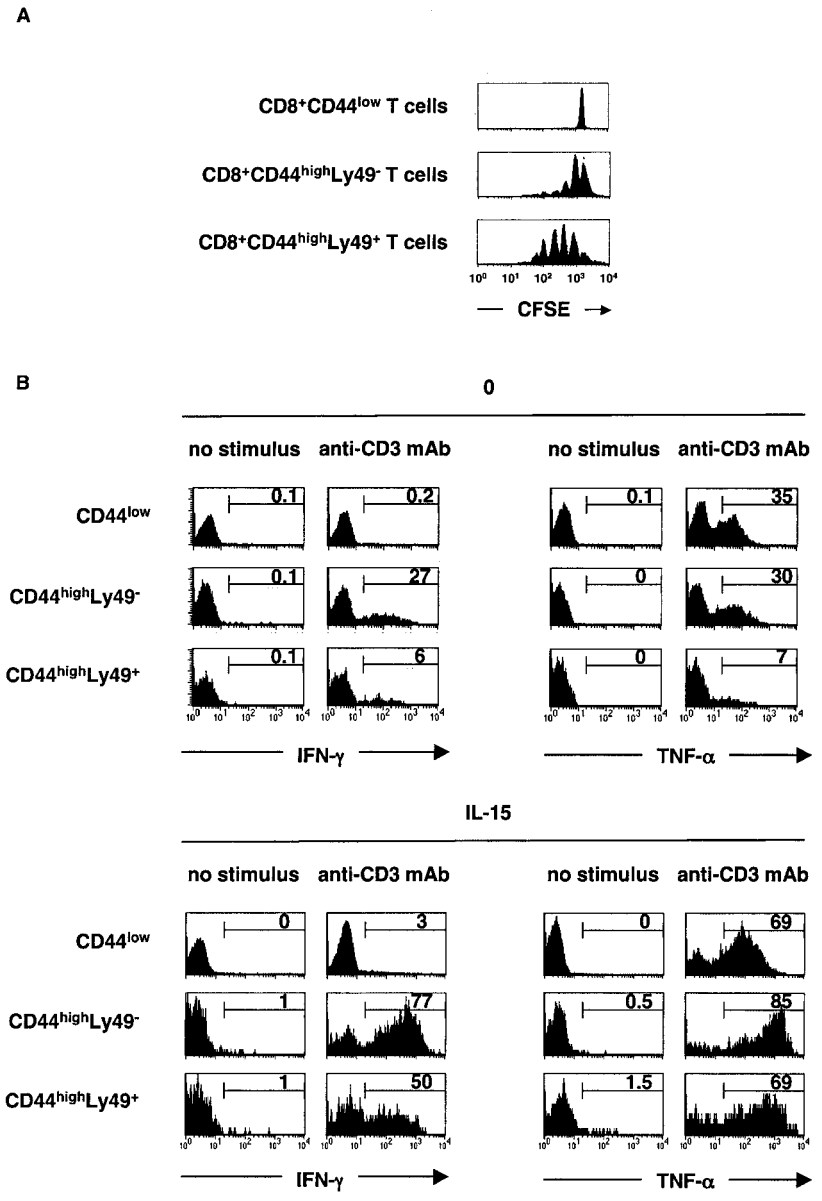

FIGURE 5. Bystander stimulation of Ly49$^+$CD8$^+$ T cells. *A*, Proliferation of indicated T cell subsets was assessed using CFSE in the presence or absence of IL-15 (100 ng/ml). Results are representative from three independent experiments conducted with groups of two or more mice. *B*, Splenocytes were cultured for 3 days in the presence or absence of IL-15 (100 ng/ml). Cells were then stimulated for 6 h by plate-bound anti-CD3 mAb in the presence of brefeldin A. Production of IFN-γ and TNF-α by indicated cell subsets was then assessed by flow cytometry. Indicated numbers represent cell percentages in marked regions. Results are representative from three independent experiments conducted with groups of two or more mice.

and as to the role of the CD8$^+$ T cell microenvironment in the shaping of this heterogeneity.

First, Ly49$^+$CD8$^+$ T cells can be distinguished from Ly49$^-$CD44^{high}CD8$^+$ T cells by their lower level of cell surface CD8β and CD3ϵ, as well as with their higher cell surface levels in CD122. In addition, Ly49$^+$CD8$^+$ T cells are enriched in T cells that share NK cell phenotypic features, such as the cell surface expression of NKG2D and CD49b. Yet, unlike NK cells, freshly isolated Ly49$^+$CD8$^+$ T cells are unable to exert natural cytotoxicity against YAC-1 tumors cells or against the MHC class I$^-$

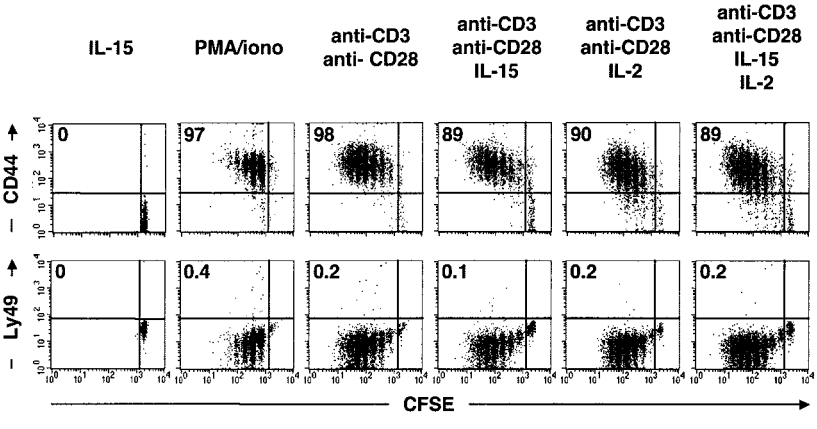

FIGURE 6. Lack of Ly49$^+$CD8$^+$ T cell induction in vitro. Naive CD44^{low}CD8$^+$ T cells were sorted from 12-wk-old mice and labeled with CFSE. These cells were then cultured for 6 days in the presence of indicated stimuli. Anti-CD3 mAb was immobilized at 10 μg/ml. Anti-CD28 mAb, PMA, ionomycin, IL-2, and IL-15 were used in solution at 1 μg/ml, 5 ng/ml, 0.5 μg/ml, 1000 U/ml, and 100 ng/ml, respectively. Before FACS analysis, cells were stained with a mix of anti-Ly49 and anti-CD44 mAb. Results are representative from three independent experiments conducted with groups of two or more mice.

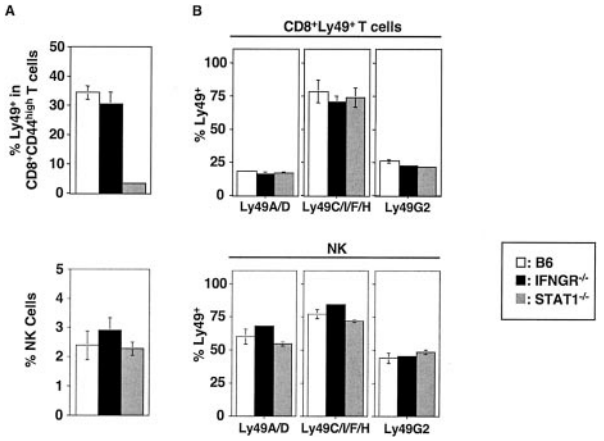

FIGURE 7. Role of STAT1 in the size of the Ly49$^+$CD8$^+$ T cell subset. *A*, Lymphocytes were isolated from the spleens of 9-mo-old wild-type, IFN-γR$^{-/-}$, and STAT1$^{-/-}$ mice and then analyzed for the percentage of Ly49$^+$ (A/D, C/I/F/H, and G) T cells present within the CD44^{high}CD8$^+$ T cell subset (*upper panel*) as well as the percentage of NK cells, defined as TCRβ^-NK1.1$^+$, present within the splenic lymphocyte population (*lower panel*). Results are expressed as mean ± SD of three mice per group. One experiment representative of three is shown. *B*, Splenic lymphocytes isolated from 9-mo-old wild-type, IFN-γR$^{-/-}$, and STAT1$^{-/-}$ mice were analyzed for the Ly49 repertoire of the Ly49$^+$CD44^{high}CD8$^+$ T cell (*upper panel*) and NK cell (*lower panel*) populations. Results are expressed as mean ± SD of individual Ly49 among total Ly49$^+$ cells (three mice per group). One experiment representative of two is shown.

RMA/S lymphoma cells. Second, it is known that central memory and effector memory cell subsets of CD8$^+$ T cells home to distinct sites (41). In contrast to cytolytic effector and effector/memory of CD8$^+$ T cells, Ly49$^+$CD8$^+$ T cells are broadly distributed in secondary lymphoid organs and are poorly represented in Peyer's patches as well as in peripheral nonlymphoid tissues such as small intestine epithelia, kidney, and lung. Third, at steady state, Ly49$^+$CD8$^+$ T cells are poor TNF-α and IFN-γ producers upon 6 h TCR triggering. In contrast, memory CD8$^+$ T cells are characterized by their ability to readily produce cytokine in this experimental setting (42, 43). This defect in cytokine production appears independent of the engagement of inhibitory Ly49 molecules on T cells because it is not restored by anti-MHC class I mAb. Yet, it is possible that inhibitory Ly49 molecules control T cell activation in a ligand-independent manner, as shown for inhibitory KIR (11). Similarly, freshly isolated human KIR$^+$CD8$^+$ T cells are cytolytic effectors with a reduced capacity to produce cytokines in the absence of apparent KIR engagement (data not shown). Along this line, freshly isolated KIR$^+$CD56^{dim} NK cells present a higher cytolytic potential and are poor cytokine producers as compared with KIR$^-$CD56^{bright} NK cells (44). These data suggest that the acquisition of cell surface inhibitory MHC class I receptors on mouse and human T cells as well as on human NK cells might correlate with their commitment into cytolytic cells at the cost of their ability to produce cytokines. This model of a CD8$^+$ T cell developmental program would support previous data showing that perforin expression and production of IFN-γ or TNF-α in CD8$^+$ T cells are largely segregated upon staphylococcal enterotoxin B stimulation in vitro (45). Yet, it is important to note that the defect in cytokine production observed with freshly isolated Ly49$^+$CD8$^+$ T cells under steady state conditions is not absolute or definitive. Indeed, no major reduction in IFN-γ or TNF-α production is observed when Ly49$^+$CD8$^+$ T cells stimulated using PMA plus ionomycin are compared with Ly49$^-$CD44^{high}CD8$^+$ T cells (data not shown). In addition, IFN-γ and TNF-α production upon TCR triggering can be partially restored by treatment with cytokines such as IL-15, providing a possible explanation for the previously reported production of IFN-γ and GM-CSF by IL-2-stimulated Ly49$^+$CD8$^+$ T cells (46). Along this line, the HY-specific Ly49$^+$CD8$^+$ T cells that expand in male mice upon adoptive transfer harbor a CD69$^+$CD62L$^-$ phenotype and readily produce IFN-γ upon 6 h TCR triggering (data not shown). Therefore, under steady state conditions Ly49$^+$CD8$^+$ T cells exhibit some anergic features (i.e., poor cytokine producers upon TCR triggering), which can be reversed by the microenvironment.

A major issue of our report resides in the first dissection of the mechanisms that lead to the Ag-specific expansion of Ly49$^+$CD8$^+$ T cells. Consistent with previous results (28), we failed to identify the conditions of Ly49 induction on CD8$^+$ T cells in vitro, despite the use of TCR engagement in the presence or absence of IL-2 and/or IL-15. One study reports the induction of Ly49 on CD8$^+$ T cells in vitro by IL-2, IL-4, or IL-15 stimulation in the absence of TCR stimulation (47). The reasons for the discrepancy with these results are unclear. In vivo, expansion of Ly49$^+$ Ag-specific T cells has not been detected in mice infected with viruses (e.g., Ad5 adenovirus, LCMV) or intracytoplasmic bacteria (*L. monocytogenes*) (8, 28), with the exception of the reported induction of Ly49 on influenza-specific T cells (48). We confirmed the lack of induction of Ly49 on K^d-restricted Ag-specific T cells upon *L. monocytogenes* challenge and extended the observation to H2-M3-restricted response upon primary or secondary stimulation in vivo. Ly49$^+$CD8$^+$ T cells have some attributes of Ag-experienced T cells, such as the frequent cell surface expression of NKG2D. Yet, we could not document an expansion of Ly49$^+$CD8$^+$ T cells upon HY-specific T cell response in TCR-β HY female mice. Using adoptive transfer of female HY-specific naive CD8$^+$ T cells into male mice, in the presence of female HY-specific CD4$^+$ T cells, we observed a time-dependent increase in the size of the Ly49$^+$CD8$^+$ T cell subset within Ag-specific cells. At present, we cannot rule out that the increase in the size of the Ly49$^+$CD8$^+$ T cell subset in our adoptive transfer model is the result of an expansion of pre-existing Ly49$^+$CD8$^+$ T cells rather than an induction of Ly49 molecules on CD8$^+$ T cells.

The still undefined instructional signals that CD4$^+$ T cells give to CD8$^+$ T cells for the generation of Ly49$^+$CD8$^+$ T cells fits with the absence of Ly49$^+$CD8$^+$ T cells in MHC class II$^{-/-}$ mice (4). The role of CD4$^+$ T cells in the generation of memory CD8$^+$ T cells has been recently revisited (49). It is clear that CD4$^+$ T cell help is critical for the induction of bona fide memory CD8$^+$ T cells (50). Our data provide the demonstration that CD4$^+$ T cells also participate in the shaping of CD8$^+$ T cell heterogeneity by promoting the induction of the Ly49$^+$CD8$^+$ T cell subset. This role of CD4$^+$ T cells in CD8$^+$ T cell maturation is supported by the altered LCMV-specific CD8$^+$ T cell response in CD4$^{-/-}$ mice (51).

Using the HY-specific adoptive transfer model, we also present here evidence that the size of the Ly49$^+$CD8$^+$ T cell subset can be the consequence of a persistent antigenic exposure of CD8$^+$ T cells. Yet, it is striking that the persistent viral infection induced by LCMV clone 13 failed to promote Ly49 expression of LCMV-specific cells (28). The microbial nature of the antigenic stimulation in the latter experimental setting might be involved in the differential Ly49 induction on Ag-specific CD8$^+$ T cells. A hallmark of the innate immune response to microbes resides in the sensing of pathogens by evolutionary conserved receptors, such as the TLRs (52). Interestingly, no reduction in the size of the Ly49$^+$CD8$^+$ T cell subset is observed in mice deficient for the two major TLR signaling adaptors, MyD88 and Lps2/Trif/TICAM-1 (data not shown). These data, combined with the absence of Ly49 expression on various virus- or bacteria-specific T cells (this report

and Ref. 28), suggest that chronic stimulation in the absence of microbial danger signals favors the emergence of $Ly49^+CD8^+$ T cells. This hypothesis is supported by the induction of Ly49A on alloreactive mouse $CD8^+$ T cells upon persistent exposure to the Ag (53), as well as by the KIR^+CD8^+ T cell expansions upon bone marrow transfer in human (54). Our results obtained in the HY-specific model are also consistent with the recently reported induction of Ly49 on self-reactive $CD8^-$ T cells (55). Yet, it remains to be investigated whether the induction of $Ly49^+$ on T cells is restricted to self-reactive T cells, or whether these circumstances of persistent stimulation also occur upon exogenous challenges. Consistent with our data, it has been recently described that chronic exposure to specific glycolipid Ag led to the expansion of CD1d-restricted invariant NKT cells with increased Ly49 expression that resulted in impaired responsiveness (56).

Finally, previous studies have shown that $Ly49^+CD8^+$ T cells are absent from IL-$15^{-/-}$ and IL-$15R\alpha^{-/-}$ mice (28, 30), and that IL-15 act as a survival factor for memory phenotype T cells, including $Ly49^+CD8^+$ T cells (57). We confirm that $Ly49^+CD8^+$ T cells are highly susceptible to IL-15-induced proliferation and reveal that TCR-induced cytokine production by these cells was also dependent upon IL-15-dependent signals. In addition, our data show that $Ly49^+CD8^+$ T cells are absent from $STAT1^{-/-}$ mice. Interestingly, $STAT1^{-/-}$ mice have normal $CD8^+$ T cell numbers and mount efficient antiviral $CD8^+$ T cell responses (58), suggesting that the $CD8^+$ T cell defect observed in these mice is selective to the $Ly49^+CD8^+$ T cell subset. At present, we cannot rule out the possibility that STAT-1-dependent signals other than those induced by type I IFN are also involved in the $Ly49^+CD8^+$ T cell defect observed in $STAT1^{-/-}$ mice. However, these data are consistent with a model in which the $CD8^+$ T cell microenvironment via type I IFN inducers leads to IL-15 production which in turn acts as an activator and a survival factor for $Ly49^+CD8^+$ T cells. Given the potential expression of Ly49 on self-reactive T cells, it is thus tempting to speculate that microbial stimulation could contribute to the proliferation and activation of pre-existing self-reactive $Ly49^+CD8^+$ T cells, consistent with the well known but still poorly understood autoimmune syndromes provoked by infection (59).

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