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Cutting Edge: Stimulation with the Cognate Self-Antigen Induces Expression of the Ly49A Receptor on Self-Reactive T Cells Which Modulates Their Responsiveness¹

Leslie Saurer,^{2*} Inge Seibold,^{*} Claudio Vallan,^{*} Werner Held,[†] and Christoph Mueller^{3*}

NK cell self-tolerance is maintained by inhibitory receptors specific for MHC class I molecules. Inhibitory NK receptors are also expressed on memory CD8 T cells but their biological relevance on T cells is unclear. In this study, we describe the expression of the Ly49A receptor on a subset of autoreactive T cells which persist in mice double-transgenic for the lymphocytic choriomeningitis virus-derived peptide gp33 and a TCR $\alpha\beta$ specific for the gp33. No Ly49A-expressing cells are found in TCR $\alpha\beta$ single-transgenic mice, indicating that the presence of the autoantigen is required for Ly49A induction. Direct evidence for an Ag-specific initiation of Ly49A expression has been obtained in vitro after stimulation of autoreactive TCR $\alpha\beta$ T cells with the cognate self-Ag. This expression of Ly49A substantially reduces Ag-specific activation of autoreactive T cells. These findings thus suggest that autoantigen-specific induction of inhibitory NK cell receptors on T cells may contribute to peripheral self-tolerance. The Journal of Immunology, 2003, 171: 0000–0000.

Natural killer cells are phenotypically defined by the expression of specific markers such as NK1.1 and DX5. Most NK cells in mice further express one or more of the nine members of the lectin-like Ly49 family of receptors (1). These receptors specifically recognize MHC class I molecules and can transduce activating or inhibitory signals upon ligand binding (2). It is believed that inhibitory Ly49 receptors actively prevent NK cells from attacking host cells with normal levels of MHC molecules and thus are a primary determinant of NK cell self-tolerance (3). Recent studies have identified the presence of NK cell markers and inhibitory Ly49 receptors also on MHC class I-restricted CD8 T cells (4–7). Expression of these molecules appears to be confined to T cells with a memory phenotype and in some cases is up-regulated during viral infections (4–7), indicating that Ag-mediated signals may initiate expres-

sion of NK markers and receptors on CD8 T cells. Although the function of the Ag recognized by the mAb DX5 (CD49b; Ref. 8) on MHC class I-restricted T cells (and NK cells) remains unclear, several reports have shown that apart from controlling NK cell activation, inhibitory Ly49 receptors also can down-modulate T cell effector functions in vitro and in vivo (9–13). Accordingly, it has been hypothesized that inhibitory NK receptors may be induced in CTL responding to self-Ags as a mechanism to maintain peripheral tolerance.

Because of the low frequency and mostly unknown Ag specificity of naturally occurring CD8 T cells expressing inhibitory Ly49 receptors, however, most of these studies have used mice transgenic (tg) for the prototypic inhibitory receptor Ly49A or Ly49A-expressing T cell lines and hybridomas (9–13). Hence, it may be difficult to deduce a physiological role for the expression of inhibitory NK receptors by CD8 T cells from these findings. In the present study, we have identified a substantial fraction of DX5- and Ly49A-expressing cells among autoreactive T cells persisting in recombination-activating gene (RAG)⁴ 2-deficient (RAG2^{-/-}) mice tg for the lymphocytic choriomeningitis virus (LCMV)-derived peptide gp33 and a TCR $\alpha\beta$ specific for the gp33. Importantly, we show that the expression of the Ly49A receptor can be induced on self-specific Ly49A⁻ T cells upon stimulation with the cognate self-Ag in vitro. Our results thus provide circumstantial evidence that stimulation with self-Ags may initiate the expression of inhibitory NK cell receptors on MHC class I-restricted T cells.

Materials and Methods

Mice

C57BL/6 mice tg for the LCMV gp33-specific TCR $\alpha\beta$ (line 318) (14) and C57BL/6 mice tg for the LCMV gp33 (line H8) (15) were provided by H. Hengartner and R. M. Zinkernagel (University Hospital, Zurich, Switzerland) and were backcrossed to RAG2^{-/-} mice. 318 \times RAG2^{-/-} and H8 \times RAG2^{-/-} mice were then intercrossed to obtain double-tg 318 \times H8 \times RAG2^{-/-} mice. All mice were kept under specific pathogen-free conditions in the core animal facility of the Medical School, University of Bern.

*Institute of Pathology, Division of Immunopathology, University of Bern, Bern, Switzerland; and [†]Ludwig Institute of Cancer Research, University of Lausanne, Lausanne, Switzerland

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² Current address: Department of Pathology and Microbiology, School of Medical Sciences, University of Bristol, Bristol, U.K.

³ Address correspondence and reprint requests to Dr. Christoph Mueller, Division of Immunopathology, Institute of Pathology, University of Bern, Murtenstrasse 31, CH-3010 Bern, Switzerland. E-mail address: christoph.mueller@pathology.unibe.ch

⁴ Abbreviations used in this paper: RAG, recombination-activating gene; LCMV, lymphocytic choriomeningitis virus; KIR, killer inhibitory receptor; tg, transgenic.

mAbs and gp33 tetramers

Fluorescence- or biotin-conjugated mAbs and purified mAb used in this study were either purchased from BD PharMingen (San Diego, CA; CD8 α (3-6.7), CD8 β (53-5.8), V α 2 (B20.1), V β 8 (MR5-2), CD44 (IM7), CD69 (H1.2F3), CD122 (TM- β 1), pan NK cell marker (DX5), NK1.1 (PK136), Ly49A (A1), Ly49C/I (SW5E6), and Ly49G2 (4D11)) or purified by protein G columns from supernatants of hybridomas originally obtained from American Type Culture Collection (Manassas, VA) and subsequently labeled according to standard protocols (TCR $\alpha\beta$ (H57-597), CD62L (Mel14)). The anti-Ly49A mAb (JR9-318) was purified and conjugated using standard procedures (16). Soluble H-2D^b tetramers complexed with biotinylated β_2 -microglobulin and gp33 peptide were kindly provided by P. Guillaume (Ludwig Institute, Lausanne, Switzerland).

Ly49A induction in vitro

T cells were sorted on a FACSVantage SE (BD Biosciences, San Jose, CA) according to the expression of DX5. In some experiments, DX5⁺ cells were further fractionated into Ly49A⁺ and Ly49A⁻ subsets. Purified T cells were cultured in 96-well round-bottom microtiter plates (Costar, Cambridge, MA) at 5×10^4 cells/well along with 3×10^5 RMA cells (H-2D^b) labeled with 2 μ g/ml gp33 peptide or the H-2D^b-binding irrelevant adn5 peptide in a total volume of 200 μ l of IMDM, supplemented with 5% FCS. After 6, 8, or 10 h, cells were stained for the expression of Ly49A, DX5, and V α 2. For analysis of mRNA expression, cells were harvested from several wells after 6 h and resorted for DX5⁺ cells. Sorted cells ($1-2 \times 10^5$) were immediately resuspended in 1 ml of Tri-reagent (Molecular Research Center, Cincinnati, OH).

Ly49A-mediated inhibition in vitro

T cells were sorted according to the expression of DX5. Purified DX5⁺ T cells were incubated in 96-well microtiter plates at 5×10^4 cells/well along with 1×10^5 RMA cells (H-2D^b) or with H-2D^d-transfected RMA cells (H-2D^{d/b}), labeled with 2 μ g/ml gp33 or adn5 peptide, in the presence of 10 μ g/ml of the blocking anti-Ly49A mAb A1 or an isotype control (mouse IgG2a). Percentage of CD69-expressing cells was determined after a 6-h culture period.

RNA isolation and RT-PCR

RNA was isolated according to the manufacturer's (Molecular Research Center) directions. RNA was DNase digested and reverse transcribed using a commercial cDNA kit (Promega, Madison, WI) and following the manufacturer's suggested conditions. cDNA was amplified for 1 min at 92°C, 1 min at 55°C, and 1 min at 72°C with 35 cycles and using the following primers: GGAGGT CACTTATTCATGG (Ly49A, sense), GCAGAACCAGTATACTTTAT (Ly49A, antisense), TGGAATCCTGTGGCATCCATGAAA (actin, sense), and TAAACGCAGCTCAGTAACAGTCC (actin, antisense).

Results and Discussion*Persisting T cells in Ag-bearing TCR tg mice express the NK cell marker DX5 but not NK1.1*

In $318 \times \text{RAG2}^{-/-}$ mice, which express a tg TCR $\alpha\beta$ (318) specific for the LCMV-derived immunodominant peptide gp33, all peripheral T cells are CD8 $\alpha\beta$ ⁺ and express the positively selected MHC class I-restricted (H-2D^b) tg TCR, as determined by flow cytometric staining with mAbs against V α 2 and V β 8 or with soluble MHC D^b tetramers (Fig. 1). Intriguingly, when in $318 \times \text{RAG2}^{-/-}$ mice the gp33 Ag (H8) is co-expressed ubiquitously under the control of a MHC class I promoter ($318 \times \text{H8} \times \text{RAG2}^{-/-}$ (Ag⁺) mice), T cells bearing the tg TCR remained present in peripheral lymphoid organs at comparable frequencies and absolute numbers as observed in $318 \times \text{RAG2}^{-/-}$ (Ag⁻) mice. The expression level of the TCR complex was generally not reduced on these CD8-negative T cells as shown by staining with either TCR V α 2- and V β 8-specific Abs, or with soluble MHC D^b tetramers, loaded with gp33 (Fig. 1A).

Hence, these autoreactive T cells that persist in the presence of the autoantigen in Ag⁺ mice differ in their TCR expression level from those observed in CD8 $\alpha\beta$ TCR $\alpha\beta$ tg T cells that were adoptively transferred into H8 mice where cell surface expression of TCR was reduced 4- to 5-fold (15). However, in our

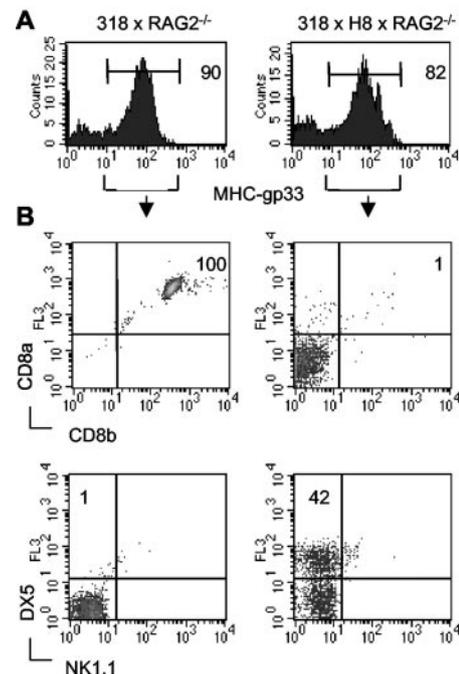


FIGURE 1. Persisting T cells in Ag⁺ mice express the NK cell marker DX5 but not NK1.1. Spleen cells isolated from $318 \times \text{RAG2}^{-/-}$ (Ag⁻) mice, and $318 \times \text{H8} \times \text{RAG2}^{-/-}$ (Ag⁺) mice, respectively, were stained with MHC-gp33 tetramers for expression of the tg TCR (A). Expression of the CD8 coreceptor and the NK cells markers NK1.1 and DX5 was assessed by gating on TCR tg cells (B). Numbers indicate the frequency of TCR tg cells within the lymphoid compartment (A) and of CD8 $\alpha\beta$ ⁺, NK1.1⁺, and DX5⁺ T cells among TCR tg cells (B). Staining is representative of at least five independent experiments.

system the majority of TCR tg T cells in secondary lymphoid organs of Ag⁺ mice completely lacked CD8 expression (Fig. 1B). Instead, a large proportion ($39 \pm 6\%$; $n = 10$) of these double-negative TCR tg T cells in the spleen of Ag⁺ mice expressed the pan NK marker DX5. The other pan NK cell marker, NK1.1, however, was never expressed on tg TCR $\alpha\beta$ -expressing cells (Fig. 1B) although it was readily detected on CD3⁻ NK cells from the same animals (data not shown). DX5⁺NK1.1⁻ TCR tg T cells were also found at high frequency ($60 \pm 5\%$; $n = 6$) in the mesenteric lymph nodes of Ag⁺ mice, but could not be detected in the thymus or the intestinal mucosa (data not shown), and, importantly, were almost completely absent from spleen (Fig. 1B) and mesenteric lymph nodes (data not shown) of Ag⁻ mice (Fig. 1B). Thus, the presence of the cognate self-Ag in LCMV-gp33 TCR tg mice resulted in the disappearance of cell surface-bearing CD8 $\alpha\beta$ T cells, but also led to the generation of a double-negative TCR tg T cell population with prominent expression of the DX5 Ag. Based on their usage of the gp33-specific, MHC class I-restricted, V α 2 V β 8 TCR, their NK1.1⁻ phenotype and their tissue distribution, however, it seems unlikely that these CD8⁻DX5⁺ T cells belong to the NK T cell subset which expresses a CD1d-restricted TCR and accounts for $\sim 15\%$ of mature thymocytes but $< 1\%$ of splenocytes (17).

Memory phenotype TCR tg T cells are contained within the DX5⁺ subset in Ag⁺ mice

Further indicative of an impact of the cognate self-Ag, TCR tg T cells from Ag⁺ mice displayed a characteristic memory phenotype with high expression levels of CD122 and CD44, but

no expression of the early activation markers CD69 and CD25 (Fig. 2A). Since the expression pattern of CD62L by TCR tg T cells from Ag⁺ mice (Fig. 2A) very much resembled the expression profile of the DX5 Ag (Fig. 1B), we compared DX5⁺ and DX5⁻ TCR tg T cells for differential expression of cell surface markers. The DX5⁺ cells were indeed mainly of the CD62L^{high} phenotype (Fig. 2B). Moreover, DX5⁺ TCR tg T cells displayed yet higher levels of CD122 and CD44 than their DX5⁻ counterparts (Fig. 2B).

DX5⁺ TCR tg T cells coexpress the Ly49A receptor

These findings strongly indicated that the expression of the DX5 Ag by self-reactive T cells in Ag⁺ mice might be related to the presence of the specific self-Ag, in particular, since in previous studies the expression of NK markers and receptors by MHC-restricted T cells also was associated with a preceding activation of T cells in vivo (4–7). To assess whether, apart from the expression of the DX5 Ag, persisting TCR tg T cells in Ag⁺ mice had indeed further acquired expression of inhibitory NK receptors, spleen cells from Ag⁺ mice were stained with mAbs against Ly49A, Ly49C/I, and Ly49G2. No distinct expression of Ly49C/I or Ly49G2 was detected on DX5⁺ T cells by FACS staining (data not shown). However, the Ly49A receptor was in fact expressed by approximately a quarter ($24.5 \pm 5.5\%$; $n = 10$) of splenic DX5⁺, but not by DX5⁻ TCR tg T cells (Fig. 3A). The differential expression of Ly49 family members with inhibitory functions on DX5⁺CD8⁻ TCR $\alpha\beta$ cells may be related to the previously reported T cell factor 1-regulated expression of Ly49A, but not of Ly49C/I and Ly49G2, on NK cells from C57BL/6 mice (18).

In mice, expression of inhibitory Ly49 receptors has been previously reported on memory-type CD8 T cells, which sim-

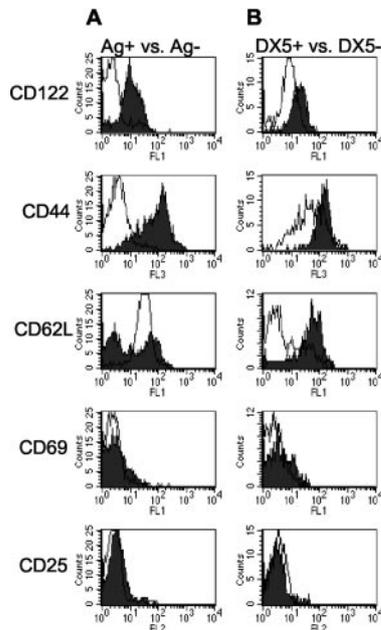


FIGURE 2. Memory phenotype TCR tg T cells are contained within the DX5⁺ subset in Ag⁺ mice. Spleen cells isolated from 318 × RAG2^{-/-} (Ag⁻) mice and 318 × H8 × RAG2^{-/-} (Ag⁺) mice, respectively, were stained with the indicated mAbs. Expression of memory and activation markers is shown as gated on TCR tg T cells from Ag⁺ (filled histograms) and Ag⁻ (lines) mice, respectively (A), or as gated on DX5⁺ (filled histograms) and DX5⁻ (lines) cells, respectively, from Ag⁺ mice (B). Staining is representative of at least three experiments.

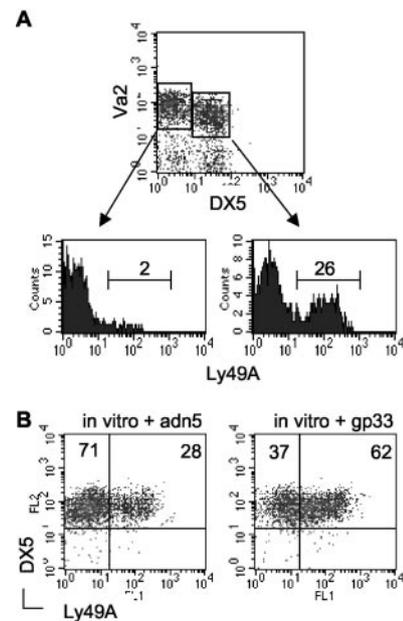


FIGURE 3. Expression of the Ly49A receptor by DX5⁺ TCR tg T cells from Ag⁺ mice. A, Spleen cells isolated from 318 × H8 × RAG2^{-/-} (Ag⁺) mice were stained with mAbs against Ly49A, DX5, and Va2. Expression of Ly49A is shown as gated on DX5⁻ (left histogram) and DX5⁺ (right histogram) TCR tg T cells, respectively. Numbers indicate the percentage of Ly49A⁺ cells between DX5⁺ and DX5⁻ TCR tg cells, respectively. Staining is representative of at least 10 experiments. B, Sorted DX5⁺ cells from pooled spleen cells of four to six 318 × H8 × RAG2^{-/-} mice were stimulated with RMA cells labeled with either the specific gp33 peptide or the irrelevant H-2D^b-binding peptide adn5. After 6 h, cells were harvested and stained for Ly49A. Numbers indicate the percentage of DX5⁺ cells negative or positive for Ly49A expression, respectively. Experiment was repeated twice with similar results.

ilar to the DX5⁺CD8⁻ TCR tg T cells found to express Ly49A in our system were also CD122 and CD44 positive and partially coexpressed the DX5 marker, but lacked CD69 expression (4). In this previous study, Ly49-expressing CD8 T cells seemed to accumulate with age. Although the authors elegantly showed that MHC class I expression on hemopoietic cells is required for the generation of Ly49-expressing CD8 T cells in secondary lymphoid organs, possibly through a bone marrow-derived APC delivering appropriate costimulatory signals, the stimuli specifically inducing Ly49 on CD8 T cells could not be defined (4). Circumstantial evidence that stimulation with self-Ags might induce inhibitory NK receptors on MHC class I-restricted T cells, however, has been provided by a study that reported expression of killer inhibitory receptors (KIR) on human CD8 T cells, specific for melanocyte differentiation Ag and isolated from healthy donors (19). Moreover, experiments using human CD8⁺ self-reactive T cell clones revealed a clear dependency of TCR triggering for sustained cell surface expression of KIR (20). To assess how stimulation with the cognate self-Ag would affect the expression of the DX5 Ag and of the Ly49A receptor on self-specific TCR tg T cells in our system, sorted DX5⁺ and DX5⁻ cells, respectively, were stimulated in vitro with RMA cells (H-2D^b) labeled with either the specific self-peptide gp33 or the H-2D^b-binding irrelevant control peptide adn5. After 6 h, a 2-fold increase in Ly49A-expressing cells was observed among DX5⁺ cells that had been stimulated in the presence of their specific self-Ag, while the frequency of adn5-stimulated Ly49A⁺ cells remained comparable to ex vivo-isolated DX5⁺ cells (Fig. 3). The expression level of the DX5 Ag

remained unchanged on DX5⁺ cells, and neither DX5 nor Ly49A appeared to be induced on the DX5⁻ subset (Fig. 3B and data not shown). Importantly, however, expression of the Ly49A receptor by DX5⁺ T cells appeared to be modulated by stimulation with the specific MHC class I-restricted self-Ag.

Expression of the Ly49A receptor is induced de novo upon stimulation with the specific self-Ag

To assess whether the observed increase in Ly49A-expressing DX5⁺ cells upon stimulation with the gp33 Ag could indeed be attributed to augmented expression of the Ly49A receptor and was not simply caused by a selective loss of Ly49A⁻ T cells, DX5⁺ T cells were fractionated into Ly49A⁺ and Ly49A⁻ subsets, respectively, for in vitro stimulation with gp33- or adn5-labeled RMA cells. As anticipated, the irrelevant adn5 peptide had no impact on the expression of Ly49A in Ly49A⁻ T cells (Fig. 4A; lines). However, stimulation with the specific self-peptide clearly induced Ly49A expression in Ly49A⁻ T cells, although expression appeared to be transient, peaking at 8 h after stimulation in vitro (Fig. 4A, filled histograms). Under these in vitro conditions, Ly49A⁺ T cells did not further up-regulate Ly49A expression upon stimulation with the gp33 Ag (Fig. 4B, filled histograms), but intriguingly, similar to previous findings made with KIR expression on human self-reactive CD8 T cell clones (20), even down-modulated expression of Ly49A when stimulated in the presence of the irrelevant control peptide (Fig. 4B, filled histograms). Hence, the increased frequency of Ly49A-expressing cells observed in Fig. 3B was in fact due to de novo surface expression of Ly49A in Ly49A⁻ T cells. In line with this notion, a clear up-regulation of mRNA for

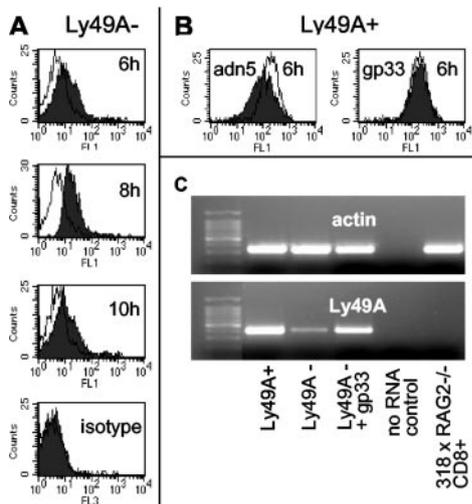


FIGURE 4. Ly49A expression is induced on DX5⁺ TCR tg T cells upon stimulation with the specific self-Ag in vitro. Pooled spleen cells obtained from four to six 318 × H8 × RAG2^{-/-} mice were separated into Ly49A⁺DX5⁺ and Ly49A⁻DX5⁺ cells, respectively, by FACS. *A*, Ly49A⁻DX5⁺ cells were stimulated with RMA cells labeled with the specific gp33 peptide (filled histograms) or the irrelevant adn5-peptide (lines). At the indicated intervals, cells were harvested and stained for the expression of Ly49A. *B*, Ly49A⁺DX5⁺ cells were stimulated as described above. After 6 h, cells were restained and expression of Ly49A was compared in cells stimulated in vitro and freshly isolated Ly49A⁺DX5⁺ cells (lines). *C*, Expression of mRNA for Ly49A was assessed in freshly isolated Ly49A⁺DX5⁺ and Ly49A⁻DX5⁺ cells, respectively, and in Ly49A⁻DX5⁺ cells, which had been stimulated with gp33-labeled RMA cells for 6 h. Experiment was repeated three times with similar results.

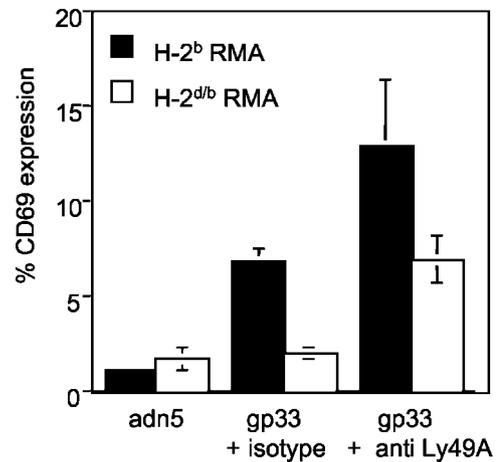


FIGURE 5. Blocking of Ly49A-MHC class I interactions results in increased Ag-specific activation of Ly49A-expressing DX5⁺ T cells. Pooled DX5⁺ splenic T cells from three donor mice were incubated with gp33, or as a control with adn5-pulsed RMA (H-2D^b) (□) or D^d-transfected RMA cells (H-2D^{d/b}) (■) in the presence of the blocking anti-Ly49A mAb A1 (+ anti-Ly49A), or an isotype control (mouse IgG2a) (+ isotype). Percentage of CD69-expressing cells was determined after a 6-h culture period. Results are mean ± SD of quadruplicates. Experiment was repeated twice with comparable results.

Ly49A was observed in Ly49A⁻ T cells stimulated with the gp33 Ag (Fig. 4C).

Blocking of Ly49A-MHC class I interactions results in increased Ag-specific activation of Ly49A-expressing DX5⁺ T cells

To assess the functional consequences of Ly49A expression on autoreactive T cells, we determined the effect of the Ly49A blocking Ab A1 on the appearance of the early activation marker CD69 on splenic DX5⁺ T cells from 318 × H8 × RAG2^{-/-} mice upon Ag-specific activation in vitro. RMA (H-2D^b) and H-2D^d-transfected RMA cells (H-2D^{d/b}), primed with gp33 or the control peptide adn5, were used as stimulator cells. As shown in Fig. 5, in the presence of the blocking anti-Ly49A mAb, the percentage of CD69-expressing DX5⁺ T cells increased 2-fold after gp33-specific activation with H-2D^b RMA cells; when H-2D^{d/b} RMA cells are used as stimulator cells an ~3-fold increase in the frequency of CD69⁺DX5⁺ T cells is seen in the presence of the anti-Ly49A mAb. Hence, the expression of the Ly49A receptor by autoreactive T cells and its functional interaction with H-2D^b ligands indeed impairs Ag-specific T cell activation. Adoptive transfers may possibly reveal the inhibitory potential in vivo of Ly49A expression on these persisting autoreactive T cells.

Although only ~25% of the ex vivo-isolated DX5⁺ autoreactive CD8⁻ T cells express Ly49A, the rapid, but transient, up-regulation of Ly49A on the majority of Ly49A DX5⁺ T cells upon autoantigen-specific activation may indicate that NKR-mediated down-modulation of T cell effector functions (9–13) may indeed contribute to the absence of immunopathological alterations in Ag⁺ mice, even after a potent LCMV infection (data not shown). Hence, autoantigen-induced expression of Ly49A may represent an additional mechanism to prevent the activation of autoaggressive T cells in the periphery.

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