Cutting Edge: Ly49A Inhibits TCR/CD3-Induced Apoptosis and IL-2 Secretion

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To evaluate the importance of Ly49A on TCR-induced cellular events, we established clones of the 1F2 T cell hybridoma expressing either Ly49A or a chimeric version, Ly49A/H, where the Ly49A cytoplasmic domain has been replaced by the Ly49H cytoplasmic domain. Ligation of Ly49A, but not Ly49A/H, with its ligand H-2D^d or anti-Ly49A mAbs caused a specific inhibition of TCR/CD3-induced IL-2 secretion. Moreover, flow cytometry analysis of hypodiploid DNA and annexin V binding revealed that ligation of Ly49A protected cells from apoptosis induced by anti-CD3 mAbs or Ag. In contrast, ligation of the Ly49A/H chimeric receptor had no antiapoptotic effect. In addition, engagement of Ly49A selectively inhibited TCR-induced Fas ligand expression whereas TCR-induced Fas expression was not significantly affected. Expression of Ly49 inhibitory receptors on T cells may represent an important mechanism for the regulation of T cell survival in vivo by inhibiting TCR-induced apoptosis and IL-2 secretion. The Journal of Immunology, 2001, 167: 6–10.

Ly49 family of receptors are C-type lectin-like molecules which bind to specific MHC class I molecules (1). They include members with inhibitory and activating function (2). The expression of activating Ly49 receptors is restricted to NK cells, whereas inhibitory Ly49 receptors are expressed on NK cells and on a small subset of T cells (3, 4). T cells expressing Ly49 correspond to the CD1-restricted NK1.1^+ T cells (5) and to non-CD1-restricted conventional T cells which are predominantly CD8^+ T cells bearing surface markers of memory phenotype (6).

Several lines of evidence suggest that Ly49 receptors may regulate TCR signaling. Transgenic expression of Ly49A down-modulates the proliferation of T cells to allogeneic stimulation (7) and impairs antiviral (8) and antitumor (9) T cell responses. Moreover, binding of Ly49A to its MHC class I ligand alters the threshold sensitivity for TCR-mediated activation (10), reduces the spontaneous IL-2 secretion in EL-4 cells (4), and inhibits CD3-induced up-regulation of CD69 in Ly49A^-CD8^- T lymphocytes (6). Expression of another member of Ly49 inhibitory receptors, Ly49G2, on lymphocytic choriomeningitis virus-specific CD8^+ T cells impairs their capacity to lyse targets that express a Ly49G2 ligand, H-2D^d (11).

In addition, using Ly49A-transgenic mice that coexpress an HMC class I ligand for Ly49A, it was demonstrated that expression of Ly49A on T cells promotes the survival of potentially self-reactive T cells by affecting both positive and negative selection of thymocytes (10, 12).

The experiments presented in this report were designed to further our understanding of the involvement of Ly49A in the regulation of T cell responses. In particular, we examined the role of Ly49A in the modulation of activation-induced cell death (AICD). We used a T cell hybridoma where TCR triggering by anti-CD3 mAbs or Ag induces AICD. Using this experimental model, we showed that engagement of Ly49A inhibits both CD3-induced IL-2 secretion and apoptosis.

Materials and Methods

Cell lines and Abs

1F2 is a T cell hybridoma specific for the I-E^p-restricted β-galactosidase (450–462) epitope (13). The CH-27-1-E^p is a B10.A-derived B cell lymphoma (H-2K^k, H-2D^d) that expresses the I-E^p class II molecule (14) and was used as APC.

mAbs used included anti-mouse CD3e, 145-2C11 (hamster Ig; American Type Culture Collection, Manassas, VA), anti-mouse Ly49A, A1 (mouse IgG2a, provided by J. Allison, University of California, Berkeley, CA), anti-H-2D^d, 34-5-8S (mouse IgG2a; American Type Culture Collection), anti-H-2K^k, 16-3-22S (mouse IgG2a; American Type Culture Collection), anti-I-E, 14-4-4S (provided by C. Daniel, Institut National de la Recherche Scientifique-Institut Armand-Frappier, Laval, Canada), and anti-Fas (Jo2; BD PharMingen, San Diego, CA).

Plasmids and transfections

The plasmid pSRα-Ly49A was generated by cloning a SacI-BamHI fragment corresponding to the Ly49A cDNA (pBSly49A, kindly provided by F. Takei, University of British Columbia, Vancouver, Canada) into the plasmid pSRαpuromycin.

The Ly49A/H construct was generated by PCR by overlap extension using the following primers and templates. Sense, 5’-CCGAATTCCGCA CCAATGAGTACCAGCAAGAGGAGG-3’ and antisense, 5’-CCAGAGCTCAGTCAATGA-3’; primers were used to amplify the cytoplasmic domain of Ly49H with Ly49H cDNA as a template (kindly provided by F. Takei, University of British Columbia). Sense, 5’-TCATTGTGATAGCTCTTGG-3’ and antisense, 5’-CGCTGAGGATCTTCAATAGGAGGAAATTATC-3’; primers were used to amplify the extracellular and transmembrane domains of Ly49A. The PCR product was subcloned into the EcoRI and BamHI sites of pSRαpuromycin. The nucleotide sequence of the chimeric construct was entirely verified.

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dGTP, dTTP), 200 nM specific oligonucleotide primers, and 2.5 U Taq Polymerase. The amplification cycles were 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min 30 s. PCR was performed for 20 cycles for β-actin and 30 cycles for Fas and Fas ligand (FasL).

**Results**

*Ly49A ligation down-modulates CD3-induced IL-2 secretion*

We generated several transfected T cell hybridoma clones expressing Ly49A or a chimeric version containing the entire extracellular and transmembrane domain of Ly49A fused to the intracytoplasmic domain of Ly49H (Ly49A/H). Ly49H is a prototype of activating Ly49 receptors. It interacts via its transmembrane region with the immunoreceptor tyrosine-based activation motif-containing molecule DAP12 and is devoid of an immunoreceptor tyrosine-based inhibition motif (ITIM) in its intracytoplasmic domain (16). Therefore, any Ly49A ITIM-mediated inhibitory signal should be absent in the Ly49A/H chimera. Multiple clones were established with the Ly49A and Ly49A/H constructs and experiments were performed with clones expressing comparable levels of TCR/CD3 at their cell surface. Stimulation of the parental and transfected cell lines with anti-CD3 mAbs coated on plastic (Fig. 1A) or on beads (Fig. 1B) induced IL-2 secretion. Coimmobilization of anti-CD3 and anti-Ly49A mAbs reduced dramatically CD3-induced IL-2 secretion in clones expressing Ly49A (Fig. 1A, and B). In contrast, IL-2 secretion was not affected by cross-linking of CD3 and the Ly49A/H chimera. This result indicates that Ly49A-mediated inhibition of IL-2 secretion likely occurs through its cytoplasmic domain. Moreover, given that the Ly49A- and Ly49A/H-transfected cells express similar levels of the Ly49A extracellular domain (data not shown), these data demonstrate that anti-Ly49A mAbs did not affect the activation of the cells by steric hindrance of TCR/CD3 cross-linking with anti-CD3 mAbs. Coengagement of Ly49A and CD3 is required to down-modulate IL-2 secretion since inhibition was not observed with anti-Ly49A and anti-CD3 mAbs coated on separate beads (Fig. 1B).

To evaluate the effect of Ly49A binding to its MHC class I ligand on IL-2 secretion, Ly49A- or Ly49A/H-expressing cells were stimulated with increasing concentrations of peptide presented by I-E^d^ on H-2^D^K^b^ APCs (Fig. 1C). Ly49A expressing cells did not secrete significant amounts of IL-2 in response to Ag...
Ly49A signaling inhibits CD3-induced apoptosis

Cross-linking with anti-CD3 and anti-Ly49A mAbs completely inhibited CD3-induced cell death in clones expressing Ly49A, whereas this treatment had no effect on clones expressing the chimeric Ly49A/H molecule (data not shown). The number of apoptotic cells was quantified by flow cytometry analysis of hypodiploid DNA and annexin V binding. In cells expressing Ly49A, treatment with anti-Ly49A mAbs diminished considerably the number of cells undergoing both early (annexin V single positive) and late (7-AAD/annexin V double positive) apoptosis as compared with cells treated with anti-CD3 mAbs alone (Fig. 2A). In contrast, a similar percentage of cells undergoing apoptosis was found in Ly49A/H-expressing cells treated or not treated with anti-Ly49A mAbs. Blocking Ly49A-ligand interaction increased significantly the percentage of T cells undergoing apoptosis, whereas the same treatment had no effect on cells expressing the chimeric Ly49A/H receptor. (Fig. 2B). These results confirm that binding of Ly49A to its ligand inhibited Apo-induced apoptosis. A marked inhibition by Ly49A of TCR-induced apoptosis was also detected by propidium iodide staining of subdiploid DNA (Fig. 3A). Fas-mediated apoptosis is potentiated by IL-2 (17, 18). Addition of IL-2 did not restore AICD when Ly49A and CD3 were coligated in clones expressing Ly49A (data not shown). Therefore, the inhibition of apoptosis by Ly49A is not the consequence of diminished IL-2 production. In addition, ligation of Ly49A failed to protect cells against glucocorticoid-induced cell death (Fig. 3B).

Discussion

Our results clearly demonstrate that inhibition of IL-2 secretion and AICD by Ly49A is mediated by the intracytoplasmic domain of Ly49A since the chimeric Ly49A/H does not deliver inhibitory signals. Ly49A contains within its cytoplasmic domain an ITIM that represents a potential binding site for Src homology 2 domain-containing phosphatases. The protein tyrosine phosphatase SHIP-1

Ly49A protects cells from AICD by inhibiting FasL expression

TCR/CD3-mediated cell death in T cell hybridomas is primarily induced through Fas (19, 20). To investigate the mechanism of Ly49A-mediated resistance to AICD, we analyzed the effect of Ly49A ligation on CD3-induced up-regulation of Fas and FasL expression (Fig. 4). Activation-induced mRNA expression of FasL was greatly reduced when CD3 and Ly49A were coligated in clones expressing Ly49A (Fig. 4A). In contrast, CD3-induced Fas mRNA and Fas cell surface expression were not affected by Ly49A cross-linking (Fig. 4). As expected, ligation of Ly49A/H did not affect CD3-induced up-regulation of Fas and FasL expression (Fig. 4). These data suggest that Ly49A inhibits AICD by preventing FasL expression. Moreover, since CD3-induced up-regulation of Fas was not inhibited by Ly49A, this result demonstrates that CD3-mediated activation events are not all negatively regulated by Ly49A.
promoter activity (23–26). In addition, it was recently shown that growth response family of transcription factors, early growth factor (1F2Ly49A) or Ly49A/H (1F2Ly49A/H) were left unstimulated (o) or were stimulated on mAb-coated plates with anti-CD3 and anti-Ly49A mAbs (CD3 + Ly49A) or anti-CD3 and control mAbs (CD3 + IgG) for 2 and 6 h. Total RNA was extracted and expression of the indicated genes was measured by RT-PCR. B, 1F2 cells expressing either Ly49A or Ly49A/H were left unstimulated (−), dotted line, or were stimulated on mAb-coated plates with anti-CD3 and anti-Ly49A mAbs (CD3 + Ly49A), thick solid line, or anti-CD3 and control mAbs (CD3 + Ig, thin solid line) for 16 h. FACS analysis of cell surface expression of Fas was performed using Jo2 Ab and FITC-labeled goat anti-hamster Ig.

FIGURE 4. Effect of Ly49A ligation on CD3-induced up-regulation of Fas and FasL expression. A, 1F2 cells expressing either Ly49A (1F2Ly49A) or Ly49A/H (1F2Ly49A/H) were left unstimulated (−) or were stimulated on mAb-coated plates with anti-CD3 and anti-Ly49A mAbs (CD3 + Ly49A) or anti-CD3 and control mAbs (CD3 + IgG) for 2 and 6 h. Total RNA was extracted and expression of the indicated genes was measured by RT-PCR. B, 1F2 cells expressing either Ly49A or Ly49A/H were left unstimulated (−, dotted line) or were stimulated on mAb-coated plates with anti-CD3 and anti-Ly49A mAbs (CD3 + Ly49A, thick solid line) or anti-CD3 and control mAbs (CD3 + Ig, thin solid line) for 16 h. FACS analysis of cell surface expression of Fas was performed using Jo2 Ab and FITC-labeled goat anti-hamster Ig.

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