

Protection from Anti-TCR/CD3-induced Apoptosis in Immature Thymocytes by a Signal through Thymic Shared Antigen-1/Stem Cell Antigen-2

By Satoshi Noda,* Atsushi Kosugi,†§ Shin-ichiroh Saitoh,*
Seiji Narumiya,* and Toshiyuki Hamaoka*

*From the *Biomedical Research Center, Osaka University Medical School, and the †School of Allied Health Sciences, Faculty of Medicine, Osaka University, Suita, Osaka 565, Japan; and ‡Precursory Research for Embryonic Science and Technology, Research Development Corporation of Japan, Kawaguchi City, Saitama Prefecture 332, Japan*

Summary

During T cell development in the thymus, the expression of thymic shared antigen-1 (TSA-1)/stem cell antigen-2 (Sca-2), a glycosylphosphatidylinositol (GPI)-anchored differentiation antigen, is developmentally regulated. The expression level of TSA-1 is the highest in most immature CD4⁻ CD8⁻ thymocytes, high in CD4⁺ CD8⁺ thymocytes, but barely detectable in mature CD4⁺CD8⁻ or CD4⁻ CD8⁺ thymocytes and peripheral T cells. We have previously shown that surface TSA-1 expression in peripheral T cells is induced upon activation and that anti-TSA-1 mAb inhibits the T cell receptor (TCR) signaling pathway in activated T cells. In the present study, we have analyzed a role of TSA-1 in thymic selection events, especially in TCR-mediated apoptosis. In *in vitro* experiments, anti-TSA-1 blocked anti-CD3-induced cell death of T cell hybridomas. When anti-TSA-1 was injected into newborn mice *in vivo* together with anti-CD3 ϵ or anti-TCR- β , TCR/CD3-mediated apoptosis of thymocytes was almost completely blocked. The blockade of apoptosis was defined by the inhibition of, first, the decrease in total number of thymocytes; second, the decrease in percentages of CD4⁺ CD8⁺ thymocytes; and third, the induction of DNA fragmentation. However, anti-TSA-1 did not block either steroid- or radiation-induced apoptosis, indicating that a signal via TSA-1 does not inhibit a common pathway of thymocyte apoptosis. Since TCR-mediated apoptosis is pivotal in thymic ontogeny, these results suggest that TSA-1/Sca-2 is an important cell surface molecule regulating the fate of a developing T cell.

Maturation of T cells in the murine thymus can be monitored by changes in expression of several cell surface markers (1). Significant progress has been made in the developmental pathway of immature thymocytes by classifying thymocytes into subpopulations on the basis of the expressions of diverse differentiation Ags. One differentiation Ag that is potentially important in T cell development is thymic shared antigen-1 (TSA-1) (2, 3). TSA-1, which is identical to stem cell antigen-2 (Sca-2) (4, 5), is a Ly-6-related protein expressed on early thymic precursor cells, immature CD3^{-/low} thymocytes, and thymic epithelial cells. The importance of this Ag as a marker in early T cell development has been defined recently. Bone marrow stem cells that home to and populate the thymus are Sca-2⁺, indicating that Sca-2 is a marker for bone marrow prothymocyte (6, 7). Although it is suggested that TSA-1 plays a regulatory role in thymocyte maturation (3, 8), the exact

function of TSA-1/Sca-2 in T cell development remains to be elucidated.

In previous studies, we have analyzed the expression and the function of TSA-1 in mature T cells (9, 10). Although freshly isolated T cells did not express TSA-1 on the cell surface, stimulation of T cells induced a marked increase of surface TSA-1 expression. These TSA-1 molecules on activated T cells have a negative regulatory role in the TCR-mediated activation pathway, since anti-TSA-1 inhibited anti-CD3-induced IL-2 production by T cell hybridomas and normal activated T cells. However, the results obtained from these *in vitro* experiments do not define a physiological role of TSA-1 *in vivo*, nor do they identify a relevance of this function of TSA-1 to T cell development.

In the present study, we investigated whether TSA-1 plays a role in thymocyte differentiation by virtue of its ability for downmodulating TCR-mediated signal transduc-

tion. To this end, we examined whether anti-TSA-1 mAb is able to inhibit TCR-mediated apoptosis in immature thymocytes. The data demonstrated that the TSA-1 inhibitory pathway is operative in blocking TCR-mediated apoptosis in vivo.

Materials and Methods

Mice and Cell Lines. Timed pregnant C57BL/6 mice were purchased from the Shizuoka Experimental Animal Laboratory (Hamamatsu, Japan). DO.11.10 is a murine T cell hybridoma (11).

mAbs and Reagents. The following Abs were used: 145-2C11 (12), anti-CD3 ϵ ; H57-597 (13), anti-TCR- β ; G7 (14), anti-Thy-1.2; and PRST1, anti-TSA-1 (9). These mAbs were purified with either Protein A- or protein G-Sepharose (Pharmacia Biotech, Inc., Piscataway, NJ) from culture supernatants. Normal hamster IgG was purchased from Cappel (Durham, NC). Hydrocortisone 21-acetate and propidium iodide were purchased from Sigma Chemical Co. (St. Louis, MO). For cell staining, FITC-conjugated anti-CD8 and phycoerythrin-conjugated anti-CD4 were obtained from Becton Dickinson (Mountain View, CA).

Irradiation. Newborn mice were irradiated with 125, 250, or 500 cGy. Irradiation was done with a GammaCell 40 equipped with a ^{137}Cs source.

Immunofluorescence Staining and FCM Analysis. Cell staining procedure and FCM analysis using FACScan (Becton Dickinson) were as described (9).

Gel Analysis of DNA Fragmentation. Thymocyte pellets (1×10^6) were resuspended in 20 μl of lysis buffer (10 mM EDTA, 50 mM Tris-HCl, pH 8.0, containing 0.5% (w/v) sodium lauryl sarkosinate and 0.5 mg/ml RNase A) and incubated at 50°C for 1 h; 10 μl proteinase K (1.5 mg/ml) were added to each sample, and incubation at 50°C was continued for another hour. The samples were then heated to 70°C, and 10 μl of 10 mM EDTA containing 1% (w/v) low melting agarose, 0.25% (w/v) bromophenol blue, and 40% (w/v) glycerol were mixed with each sample, and incubation was continued for 1 h. The samples were then loaded on a 2% (w/v) agarose gel containing 1 $\mu\text{g/ml}$ ethidium bromide in Tris-borate buffer and visualized by UV fluorescence.

Results

Inhibition of Anti-TCR-Induced Apoptosis by Anti-TSA-1 mAb in T Cell Hybridomas. Our previous study demonstrated a negative regulatory role of TSA-1 in the TCR/CD3-mediated signaling pathway as assessed by the inhibition of IL-2 production from T cell hybridomas (9). We also demonstrated that PRST1, an anti-TSA-1 mAb, exhibited an effect on T cells in the absence of accessory cells in some experiments, indicating that our mAb has the ability to deliver a signal to T cells directly (10). Since T cell hybridomas are known to undergo apoptosis following activation (15), we tried to determine whether anti-TSA-1 is capable of inhibiting the anti-CD3-induced death pathway in T cell hybridomas. As shown in Fig. 1, when T cell hybridoma DO.11.10 cells were stimulated with anti-CD3 in the presence of irradiated Thy-1-depleted spleen cells, DO.11.10 cells underwent cell death as expected. However, anti-CD3-induced cell death was clearly inhibited by the addition of PRST1. This effect was specific for PRST1, be-

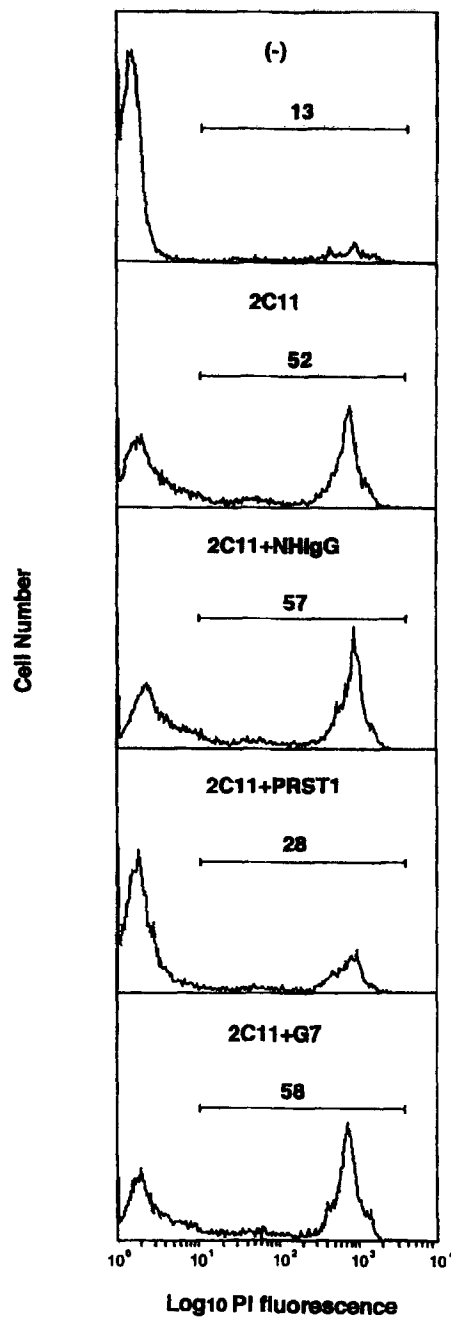


Figure 1. Inhibition of anti-CD3-induced apoptosis by anti-TSA-1 mAb in T cell hybridomas. T cell hybridoma DO.11.10 cells (5×10^5 cells per well) and 3,000-rad-irradiated Thy-1-depleted spleen cells (1×10^6 cells per well) were cultured for 12 h with the indicated Abs. The Abs used were 2C11 (anti-CD3 ϵ mAb, 50 ng/ml); NHlgG (normal hamster IgG, 10 $\mu\text{g/ml}$); PRST1 (anti-TSA-1 mAb, 10 $\mu\text{g/ml}$); and G7 (anti-Thy-1 mAb, 10 $\mu\text{g/ml}$). Cell death of T cell hybridomas was determined by propidium iodide (10 $\mu\text{g/ml}$) staining for nonpermeabilized cells. The discrimination between DO.11.10 cells and spleen cells was performed by electrical gating using forward versus side scatter dot plot. The region shown with horizontal solid line in each histogram represents the staining of dead cells and the percentage of such cells indicated.

cause normal hamster IgG and G7, a mAb against Thy-1, failed to exhibit inhibitory effects on anti-CD3-induced cell death of T cell hybridomas (Fig. 1). Thus, ligation of TSA-1 by PRST1 can protect T cell hybridomas from anti-TCR-induced apoptosis *in vitro*.

Inhibition of Anti-TCR-Induced Apoptosis by Anti-TSA-1 mAb in Neonatal Thymocytes In Vivo. We next asked whether

TSA-1 plays a role in thymocyte development by its ability to downmodulate TCR/CD3-mediated signal transduction. Since one outcome of TCR signaling in early thymocytes is clonal deletion by programmed cell death, we examined whether anti-TSA-1 is capable of inhibiting thymocyte apoptosis induced by anti-CD3 injection *in vivo*. In preliminary experiments, we analyzed doses of PRST1

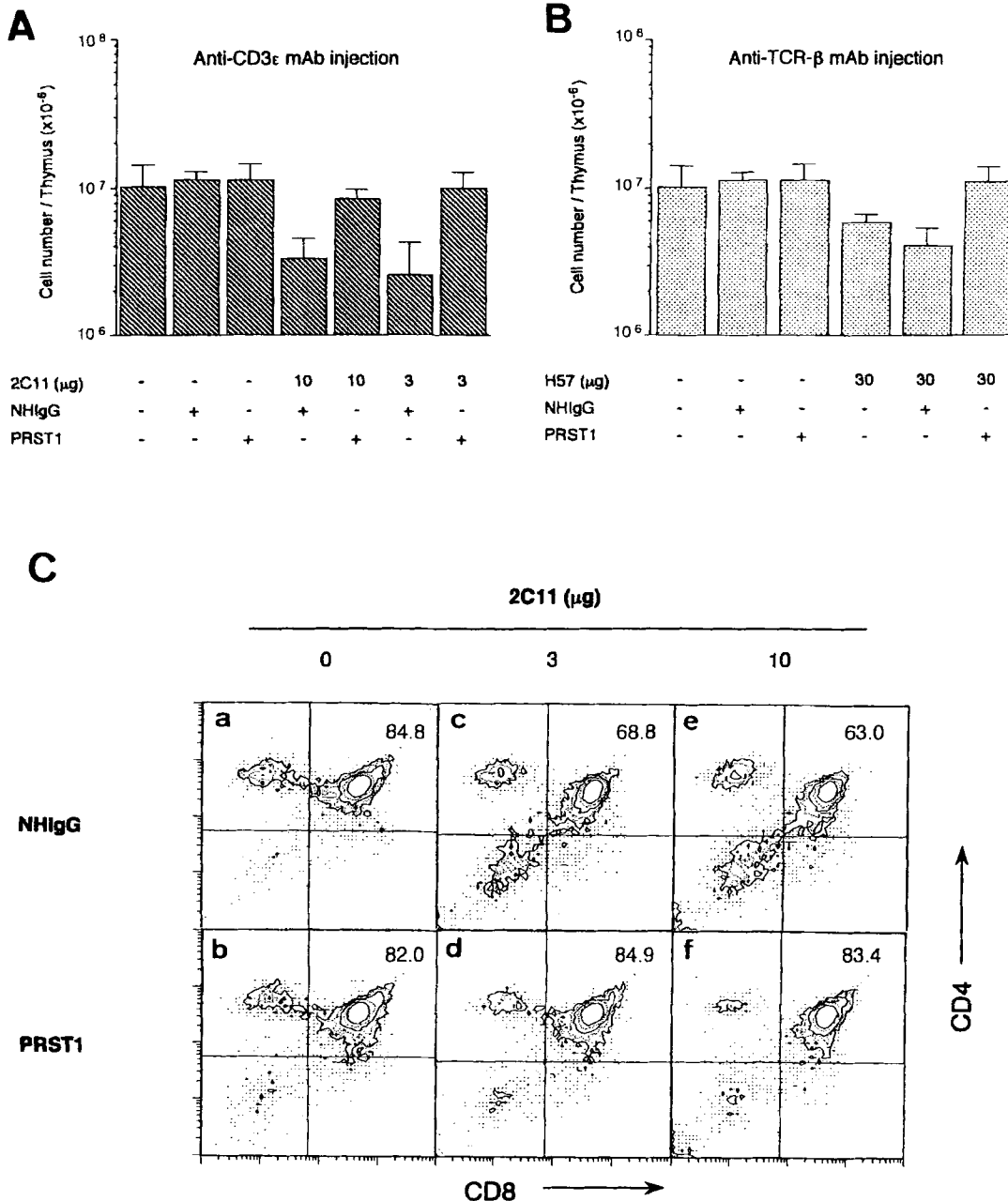


Figure 2. Rescue from anti-TCR-induced apoptosis with anti-TSA-1 mAb in neonatal thymocytes *in vivo*. (A and B) Newborn C57BL/6 mice at day 0 (within 24 h of birth) were injected intraperitoneally with 100 μ g of PRST1 or normal hamster IgG (NHlgG). After 12 h, mice were injected again with 100 μ g of PRST1 or NHlgG together with either 2C11 (anti-CD3 ϵ ; A) or H57-597 (anti-TCR- β ; B) at the dose indicated. 20 h after the injection of anti-TCR antibodies, thymuses were removed from the mice and viable cells were counted. Results are presented as mean \pm SE of 4–7 independent experiments. (C) Newborn mice were injected with 100 μ g of PRST1 or NHlgG. After 12 h, mice were injected again with 100 μ g of PRST1 or NHlgG together with 2C11 as described above. Thymocytes from these mice were stained with FITC-conjugated anti-CD8 and PE-conjugated anti-CD4 and were analyzed with FCM.

injected intraperitoneally into newborn mice. The dose used (total 200 μg per mouse administered with 100 μg per injection at day 0 and day 1 of birth) was determined by FCM analysis (data not shown).

As reported previously (16), when 3 or 10 μg of the purified anti-CD3 ϵ was injected into newborn mice, apoptotic cell death was induced in neonatal thymocytes at 16–20 h after the injection, resulting in the reduction of total thymocyte number (Fig. 2 A) and the decrease in the percentage of CD4⁺ CD8⁺ thymocytes (Fig. 2 C, panels *c* and *e*). However, cell death of thymocytes induced by anti-CD3 ϵ was dramatically inhibited when mice were injected with PRST1 along with anti-CD3 ϵ (Fig. 2 A). The inhibition of thymocyte apoptosis by PRST1 was confirmed by FCM analysis, such that the percentages of CD4⁺ CD8⁺ thymocytes from newborn mice injected with anti-CD3 ϵ plus PRST1 was not decreased in contrast with those from mice injected with anti-CD3 ϵ plus normal hamster IgG (Fig. 2 C, panels *d* and *f*). It should be noted that the injection of PRST1 alone did not affect the development of neonatal thymocytes (Fig. 2). The inhibitory effect of PRST1 was not limited to anti-CD3-induced cell death, since PRST1 was capable of inhibiting cell death of thymocytes induced by anti-TCR- β injection (Fig. 2 B). To confirm that the death of neonatal thymocytes induced by anti-TCR antibody occurred by apoptosis, and to check that the apoptosis was in fact protected by PRST1, chromosomal DNA was prepared from neonatal thymocytes from mice injected with anti-CD3 ϵ or anti-CD3 ϵ plus PRST1 and was analyzed by agarose gel electrophoresis. As shown in Fig. 3, at 20 h after the injection with 10 μg of anti-CD3 ϵ , most of the thymic DNA was degraded in the stepladder fashion that is characteristic of apoptosis. In contrast, PRST1 injection clearly

blocked the anti-CD3-induced fragmentation of chromosomal DNA. As a control, mice were injected either with anti-CD3 ϵ plus normal hamster IgG or anti-CD3 ϵ plus anti-Thy-1. These treatments did not inhibit the degradation of chromosomal DNA. Thus, these results demonstrate that engagement of TSA-1 by PRST1 is capable of inhibiting the TCR signaling pathway in immature thymocytes, resulting in protection from apoptosis *in vivo*.

Effect of Anti-TSA-1 mAb on Thymocyte Apoptosis Induced by Glucocorticoids or Irradiation. Apoptosis of thymocytes can be induced by various stimuli such as glucocorticoids or irradiation (17). We next examined whether apoptosis induced by stimuli other than TCR cross-linking can also be blocked by a signal through TSA-1. Newborn mice were injected with 100 μg of PRST1 at day 0 and day 1, and the injection of hydrocortisone or γ -irradiation was performed immediately after the second injection of PRST1. As shown in Fig. 4 A, the injection of hydrocortisone induced thymocyte apoptosis in a dose-dependent fashion. In

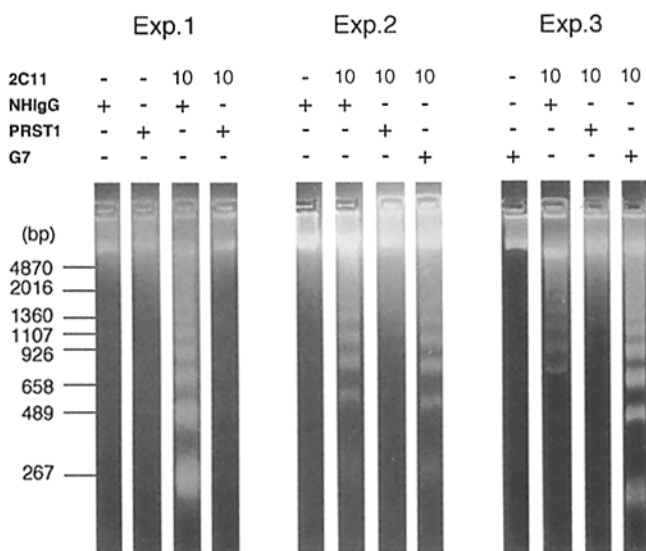


Figure 3. Agarose gel electrophoresis of DNA extracted from neonatal thymocytes of mice injected with anti-CD3 ϵ mAb and anti-TSA-1 mAb. Newborn mice were treated as described in Fig. 2. The genomic DNA was extracted and analyzed by gel electrophoresis. Molecular size standards are shown at left.

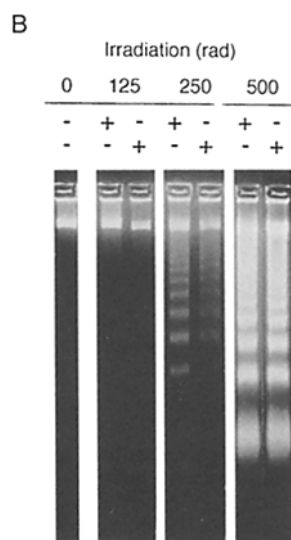
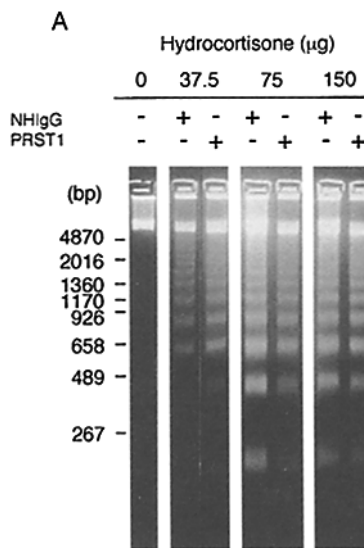


Figure 4. The effect of anti-TSA-1 mAb on steroid- or radiation-induced apoptosis in neonatal thymocytes. Newborn C57BL/6 mice at day 0 (within 24 h of birth) were injected intraperitoneally with 100 μg of PRST1 or normal hamster IgG (NHlgG). After 21 h (A) or 13 h (B), the mice were injected again with 100 μg of PRST1 or NHlgG. Mice were then injected with hydrocortisone (A) or irradiated (B) at different doses as indicated. After 8 h (A) or 12 h (B), thymuses were removed from these mice. The genomic DNA was extracted and analyzed by gel electrophoresis. Molecular size standards are shown at left.

contrast with anti-CD3-induced apoptosis, steroid-induced apoptosis was not blocked by PRST1 injection at any dose of hydrocortisone. Similarly, radiation-induced apoptosis was resistant to a signal through TSA-1 (Fig. 4 B). Thus, these results indicate that anti-TSA-1 does not inhibit a common pathway of thymocyte apoptosis, but specifically blocks TCR-mediated apoptosis.

Discussion

In a previous study, we described a mAb against TSA-1 that is capable of inhibiting anti-TCR/CD3-mediated T cell activation in T cell hybridomas (9). The further functional characterization of TSA-1 revealed that TSA-1 delivers an inhibitory signal to normal activated T cells, and that the signal through TSA-1 blocks an early signaling event in TCR/CD3-mediated T cell activation (10). The fact that thymocytes dramatically downregulate TSA-1 surface expression while maturing from CD4⁺ CD8⁺ thymocytes to CD4⁺ CD8⁻ or CD4⁻ CD8⁺ mature thymocytes led us to explore the possibility that this molecule could participate in thymic selection events by virtue of its ability for modulating TCR signaling. Since negative selection of thymocytes is generally considered to be caused by TCR-mediated apoptosis (18), we investigated in the present study whether a signal via TSA-1 is able to block apoptosis induced by cross-linking of the TCR. The data clearly demonstrated the ability of TSA-1 to deliver a signal to block anti-TCR-mediated apoptosis of immature thymocytes.

It is interesting to note that anti-TSA-1 injection in vivo did not affect positive selection in neonatal thymocytes (see Fig. 2 C). We did not observe the reduction of mature CD4⁺ CD8⁻ or CD4⁻ CD8⁺ thymocytes in mice continuously injected with PRST1 twice a week from birth (data not shown). It is thus far unknown why anti-TSA-1 does not inhibit TCR-mediated positive selection in contrast

with the inhibitory effect on TCR-mediated apoptosis. The difference between stimulation with MHC plus peptides and that with anti-TCR may account for the failure to detect an effect on positive selection. Alternatively, since the low affinity interaction between the TCR and its ligand is considered to be capable of inducing positive selection, a signal via TSA-1 may not block TCR signaling generated through such low affinity interaction.

Ly-6A/E has been recently shown, in experiments with transgenic mice, to mediate cell-cell adhesion (19). The Ly-6-related proteins may have a role in thymocyte development, since the maturation of thymocytes was severely affected in the transgenic mice overexpressing Ly-6A.2 (20). If Ly6-A.2 expressed on transgenic thymocytes has the same inhibitory effect on TCR signaling as TSA-1, the maturational arrest observed in transgenic mice could be explained by this function. In support of this, mAbs against Ly-6 proteins have been shown to exhibit an inhibitory effect on the TCR signaling pathway in mature T cells (21).

There exist several mAbs against TSA-1/Sca-2. Although we have not tested whether other anti-TSA-1/Sca-2 Abs are capable of inhibiting TCR-mediated apoptosis of thymocytes, our previous work using T cell hybridomas clearly demonstrated that our mAb, PRST1, is the only mAb that has the ability to induce downmodulation of TCR signaling (10). PRST1 could be different from other mAbs in that it recognizes a functional epitope within a TSA-1 molecule, as has been reported in other GPI-anchored proteins. Taking advantage of the property of our mAb, we investigated a potential role of TSA-1 in T cell development in vivo in this study. However, the determination of physiological significance of our findings may await identification of the natural ligand of TSA-1 in thymus. Studies are now in progress to investigate the ligand for TSA-1 using TSA-1-human IgG proteins as the probe.

The authors thank Drs. Kensuke Miyake, Yasuhiro Minami, and Masato Ogata for helpful discussions and critical review of the manuscript.

This work was supported by funds from the Osaka Foundation for Promotion of Clinical Immunology.

Address correspondence to Dr. Atsushi Kosugi, School of Allied Health Sciences, Faculty of Medicine, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565, Japan.

Received for publication 28 November 1995 and in revised form 7 February 1996.

References

1. Godfrey, D.I., and A. Zlotnik. 1993. Control points in early T-cell development. *Immunol. Today*. 14:547-553.
2. Godfrey, D.I., M. Masciantonio, C.L. Tucek, M.A. Malin, R.L. Boyd, and P. Hugo. 1992. Thymic shared antigen-1, a novel thymocyte marker discriminating immature from mature thymocyte subsets. *J. Immunol.* 148:2006-2011.
3. MacNeil, I., J. Kennedy, D.I. Godfrey, N.A. Jenkins, M. Masciantonio, C. Mineo, D.J. Gilbert, N.G. Copeland, R.L. Boyd, and A. Zlotnik. 1993. Isolation of a cDNA encoding thymic shared antigen-1, a new member of the Ly6 family with a possible role in T cell development. *J. Immunol.* 151: 6913-6923.
4. Spangrude, G.J., Y. Aihara, I.L. Weissman, and J. Klein. 1988. The stem cell antigens Sca-1 and Sca-2 subdivide thy-

- mic and peripheral T lymphocytes into unique subsets. *J. Immunol.* 141:3697–3707.
5. Classon, B.J., and L. Coverdale. 1994. Mouse stem cell antigen Sca-2 is a member of the Ly-6 family of cell surface proteins. *Proc. Natl. Acad. Sci. USA.* 91:5296–5300.
 6. Wu, L., M. Antica, G.R. Johnson, R. Scollay, and K. Shortman. 1991. Developmental potential of the earliest precursor cells from the adult mouse thymus. *J. Exp. Med.* 174:1617–1627.
 7. Antica, M., L. Wu, K. Shortman, and R. Scollay. 1994. Thymic stem cells in mouse bone marrow. *Blood.* 84:111–117.
 8. Randle, E.S., G.A. Waanders, M. Masciantonio, D.I. Godfrey, and R.L. Boyd. 1993. A lymphostromal molecule, thymic shared Ag-1, regulates early thymocyte development in fetal thymus organ culture. *J. Immunol.* 151:6027–6035.
 9. Kosugi, A., S.-I. Saitoh, S. Narumiya, K. Miyake, and T. Hamaoka. 1994. Activation-induced expression of thymic shared antigen-1 on T lymphocytes and its inhibitory role for TCR-mediated IL-2 production. *Int. Immunol.* 6:1967–1976.
 10. Saitoh, S.-I., A. Kosugi, S. Noda, N. Yamamoto, M. Ogata, Y. Minami, K. Miyake, and T. Hamaoka. 1995. Modulation of T cell receptor-mediated signaling pathway by thymic shared antigen-1 (TSA-1)/stem cell antigen-2 (Sca-2). *J. Immunol.* 155:5574–5581.
 11. Haskins, K., R. Kubo, J. White, M. Pigeon, J. Kappler, and P. Marrack. 1983. The major histocompatibility complex-restricted antigen receptor on T cells. I. Isolation with a monoclonal antibody. *J. Exp. Med.* 157:1149–1169.
 12. Leo, O., M. Foo, D.H. Sachs, L.E. Samelson, and J.A. Bluestone. 1987. Identification of a monoclonal antibody specific for a murine T3 polypeptide. *Proc. Natl. Acad. Sci. USA.* 84:1374–1378.
 13. Kubo, R.T., W. Born, J.W. Kappler, P. Marrack, and M. Pigeon. 1989. Characterization of a monoclonal antibody which detects all murine $\alpha\beta$ T cell receptors. *J. Immunol.* 142:2736–2742.
 14. Gunter, K.C., T.R. Malek, and E.M. Shevach. 1984. T cell-activating properties of an anti-Thy-1 monoclonal antibody. Possible analogy to OKT3/Leu-4. *J. Exp. Med.* 159:716–730.
 15. Ashwell, J.D., R.E. Cunningham, P.D. Noguchi, and D. Hernandez. 1987. Cell growth cycle block of T cell hybridomas upon activation with antigen. *J. Exp. Med.* 165:173–194.
 16. Shi, Y., B.M. Sahai, and D.R. Green. 1989. Cyclosporin A inhibits activation-induced cell death in T-cell hybridomas and thymocytes. *Nature (Lond.)* 339:625–626.
 17. Cohen, J.J., R.C. Duke, V.A. Fadok, and K.S. Sellins. 1992. Apoptosis and programmed cell death in immunity. *Annu. Rev. Immunol.* 10:267–293.
 18. von Boehmer, H. 1992. Thymic selection: a matter of life and death. *Immunol. Today.* 13:454–458.
 19. Bamezai, A., and K.L. Rock. 1995. Overexpressed Ly-6A.2 mediates cell-cell adhesion by binding a ligand expressed on lymphoid cells. *Proc. Natl. Acad. Sci. USA.* 92:4294–4298.
 20. Bamezai, A., D. Palliser, A. Berezovskaya, J. McGrew, K. Higgins, E. Lacy, and K.L. Rock. 1995. Regulated expression of Ly-6A.2 is important for T cell development. *J. Immunol.* 154:4233–4239.
 21. Codias, E.K., J.E. Rutter, T.J. Fleming, and T.R. Malek. 1990. Down-regulation of IL-2 production by activation of T cells through Ly-6A/E. *J. Immunol.* 145:1407–1414.