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Thrombospondin-1 Inhibits TCR-Mediated T Lymphocyte Early Activation

Zhuqing Li, Liusheng He,[1] Katherine E. Wilson,[2] and David D. Roberts[3]

Biological activities of the matrix glycoprotein thrombospondin-1 (TSP1) are cell type specific and depend on the relative expression or activation of several TSP1 receptors. Although engaging individual TSP1 receptors in T lymphocytes can elicit costimulating signals, in this study we show that intact TSP1 inhibits TCR-mediated T cell activation, assessed globally using cDNA microarrays. TSP1 signaling suppressed expression of several genes induced in Jurkat T cells, including the T cell activation markers CD69, early growth response gene-1 (Egr-1), and phosphatase of activated cells (PAC-1). TCR-stimulated and CD47-costimulated IL-2 secretion and cell surface CD69 expression were also inhibited by TSP1. The specific inhibitory effect of TSP1 was verified in freshly isolated human PBMCs. TSP1 inhibited TCR-mediated but not protein kinase C-mediated T cell activation. Using CD69 expression as a marker, we demonstrated that the inhibitory activity of TSP1 depended on two TSP1 receptors, CD47 and integrin-associated protein heparan sulfate proteoglycans. Signals from these receptors inhibited TCR signaling downstream of ZAP70, but upstream of NF-AT. Therefore, the expression of TSP1 induced during wound repair and in tumor stroma may limit T cell activation at these sites. ***The Journal of Immunology,*** **2001, 166: 2427–2436.**

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T lymphocytes play key roles in host immune responses. Activation of T cells enhances host immune responses to both foreign and self Ags. Because of its critical role in host immune responses, T cell activation is tightly regulated by secreted cytokines and chemokines as well as by cell-cell contact and cell-matrix signaling. The activation of T cells is primarily mediated by the TCR, but several important regulatory receptors have also been identified that can either costimulate or inhibit TCR signals (1–3). Interest in the negative regulation of T cell activation has been stimulated by the need to develop improved therapies to treat autoimmune diseases, AIDS, and cancer (4–6).

Interactions between T cells and extracellular matrix proteins play pivotal roles in several T cell functions, including T cell homing, recruitment to inflammatory sites (7–9), and regulation of T cell activation (10–12). Several extracellular matrix proteins, including fibronectin, collagen, vitronectin, and laminin, have been shown to influence T cell functions by inducing T cell adhesion, motility, trafficking, and T cell coactivation (12–14). However, recent data using tenascin suggest that some matrix proteins can inhibit TCR-mediated T cell activation (15). Thus, matrix proteins may have both stimulatory and inhibitory effects on T cell activation.

Thrombospondin-1 (TSP1)[4] is an extracellular matrix glycoprotein that displays distinct biological activities on different cell types. TSP1 inhibits angiogenesis and tumor growth (reviewed in Refs. 16 and 17), activates latent TGF-β (18, 19), and is necessary for maintenance of pulmonary homeostasis (20). The diverse biological effects of TSP1 have been partially attributed to the multiple functional domains of the protein that engage corresponding receptors on the surface of the targeted cells. Differential expression or activation of cell surface receptors for TSP1, including integrins, CD36, CD47, low density lipoprotein receptor-related protein, proteoglycans, and sulfatides, may dictate the specific responses of each cell type to TSP1 (16).

Several studies have suggested that TSP1 can regulate T cell function. TSP1 mediates activation-dependent T cell adhesion through binding to $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins (21). TSP1 also modulates intracellular signaling cascades in anti-CD3-activated T cells, mediated by β_1 integrins, CD47, and proteoglycans (22). Peptides from TSP1 that bind to two of these TSP1 receptors synergize with TCR activation to activate Ras and mitogen-activated protein (MAP) kinase signaling pathways (22). Functionally, TSP1 has been shown to selectively inhibit IL-12 production in monocytes via a CD47-dependent mechanism (23). However, CD47 is a costimulatory receptor on T cells (24). In addition, TSP1 null transgenic mice seem to be more susceptible to pulmonary bacterial infection, suggesting an impact of TSP1 on host immune responses (20). A recent study using an autoreactive T cell clone isolated from rheumatoid arthritis synovium reported a stimulatory activity of immobilized TSP1 (25). These observations demonstrate that TSP1 regulates T cell activation, but are consistent with both inhibitory and costimulatory activities. To clarify this issue, we have investigated the global effects of TSP1 on T cell activation and identified two TSP1 receptors that mediate its activity. We show that intact TSP1 is a potent inhibitor of TCR-mediated T cell activation. Although this inhibition is TCR specific, it is not due to direct interference with upstream signaling events of the TCR signal transduction pathway.

Materials and Methods

Antibodies

The following Abs were used: anti-human CD3 (clone HIT3a; BD PharMingen, San Diego, CA), human CD47-blocking Ab (clone B6H12.2; BD PharMingen), human CD47-stimulating Ab (clone CIKm 1; ICN, Costa

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[4] Abbreviations used in this paper: TSP1, thrombospondin-1; CAT, chloramphenicol acetyltransferase; CD27BP, CD27-binding protein; Egr, early growth response gene; FAST, Fas-activated serine/threonine kinase; HSPG, heparan sulfate proteoglycan; LAT, linker for activation of T cells; MAP, mitogen-activated protein; PAC-1, phosphatase of activated cells; PP2A, protein phosphatase 2A; TIEG, TGF-β-inducible early gene.

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Mesa, CA), PE-conjugated anti-human CD69 (Coulter Immunotech, Miami, FL), anti-ZAP70 (a general gift from Dr. Larry Samelson's laboratory, National Cancer Institute, Bethesda, MD), anti-phosphotyrosine Ab (RC 20; BD Transduction Laboratories, Lexington, KY), and activated TGF-β1-neutralizing Ab (Life Technologies, Rockville, MD).

Cell cultures and stimulation

The Jurkat T cell line (provided by Dr. Kevin Gardner, National Cancer Institute) was maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, and 1× penicillin and streptomycin (unless specified, all culture medium and medium supplements were purchased from Biofluids, Rockville, MD). Human PBMCs were prepared by gradient centrifugation. In brief, fresh human blood buffy coat (obtained from the National Institutes of Health blood bank under National Institutes of Health Multiple Project Assurance M-1000) was diluted 1/5 with sterile 1× PBS. Human PBMCs were isolated by mixing Ficoll Plus (Pharmacia, Piscataway, NJ) and the diluted buffy coat and centrifuged at $100 \times g$ at room temperature for 30 min. Isolated fresh human PBMCs were used immediately for experimental analysis. For cell stimulation, cell cultures were centrifuged and resuspended in prewarmed RPMI 1640 supplemented with 0.1% BSA (Sigma, St. Louis, MO) at the indicated cell concentrations. The cell suspensions were dispensed into six-well plates (NUNCLON; Nalge Nunc International, Rochester, NY) in the volume of 2 ml/well. Each well was precoated with or without Abs. Anti-CD3 and anti-CD47 Ab were both coated as 1 μg/well in 1× Dulbecco's PBS (pH 7; Life Technologies) at 4°C overnight. For TSP1 coating, the designated concentration of TSP1 was coated on the plate either in Dulbecco's PBS (pH 7) or in carbonate buffer (pH 9) at 4°C overnight. For soluble TSP1 treatment, TSP1 and other reagents were added in solution separately at the denoted concentrations. Cells were then incubated at 37°C with 5% CO_2 for the designated times.

The sequence of peptides that were used in this study was as follows: CD47-binding peptide 7N3 = FIRVVMYEGKK; control CD47-binding mutant peptide 604 = FIRGGMYEGKK. Amino acids were represented by single alphabetic symbols.

cDNA microarray analysis

To examine differential gene expression patterns, T cells were divided into two groups, resting T cells and anti-CD3-stimulated T cells, and cultured with or without TSP1 for the designated times. Poly(A^+) RNAs were purified from isolated cells using the FastTrack 2.0 Kit (Invitrogen, Carlsbad, CA), according to the manufacturer's instruction. For microarray studies, 2 μg of poly(A^+) RNA from each group was labeled with either Cy3 or Cy5 dye (Amersham, Piscataway, NJ) by a reverse-transcription labeling procedure. The labeled probes were then purified, and the Cy3- and Cy5-labeled probes from the two groups intended for differential gene expression analysis were then combined. The combined probe was used for hybridization. To hybridize, the 2.2K Oncochip (National Cancer Institute Microarray Facility) was carefully covered with 15 μl of the combined probe under a glass coverslip. The hybridization stringency was predetermined for optimum noise/signal ratio. The slide was sealed and incubated in a 65°C water bath for 16–20 h. The coverslip was then washed off, and the Oncochip was washed with different stringency of washing buffer. Specific hybridization of labeled cDNA to the Oncochip was quantified by scanning the Oncochip through an Avalanch Laser Scanner (Molecular Dynamics, Sunnyvale, CA). Specific signals representing differential gene expression of the two samples were analyzed using the ArraySuite Microarray analysis software provided by Molecular Dynamics. To identify potential genes with altered gene expression patterns, only those genes with more than 2-fold differences of expression and more than 500 arbitrary units of fluorescence intensity under paired control conditions were selected for further analysis.

RNase protection assay

Cells were cultured as described above. Overnight culture of T cells at concentration of 5×10^5/ml was collected and resuspended in RPMI 1640 + 0.1% BSA at concentration of 1×10^6/ml. The cell suspension was plated at 2 ml/well into six-well plates precoated with anti-CD3 Ab (1 μg/well) and incubated at 37°C for the designated times. Cells were then harvested and washed, and total RNA was isolated using TRIzol reagent (Life Technologies). The RNase protection assay was performed using the RiboQuant kit (BD PharMingen), according to the manufacturer's instruction. Briefly, equal amounts of total RNA samples were hybridized with synthesized cytokine-specific probes labeled with [^{32}P]UTP, the hybridized RNA samples were digested with RNase, and protected cytokine-specific mRNAs were resolved in sequencing SDS-PAGE gel. Specific signals were detected by autoradiography. Products for the housekeeping genes GAPDH and L32 were used as loading controls.

IL-2 ELISA

Cells were cultured as described above and stimulated in a flat-bottom 96-well plate. Culture supernatants were used to measure secreted IL-2 level using a commercially available ELISA kit (R&D Systems, Minneapolis, MN). The ELISA was performed according to the manufacturer's instruction. Briefly, 100 μl of the culture supernatant was incubated with anti-IL-2 Ab precoated on the wells. After extensive washing, bound IL-2 was detected by an HRP-conjugated second Ab. The cytokine expression level was quantified by regression analysis using IL-2 standard curve.

Flow cytometry analysis

The Jurkat T cells or freshly isolated human PBMCs, treated as described above and in the text, were isolated and washed with PBS containing 1% BSA. About 1×10^6 cells were mixed with 10 μl of PE-conjugated anti-human CD69 Ab and incubated in the dark on ice for 90 min. Unbound Abs were washed off, and the cells were fixed with 0.1% paraformaldehyde on ice for 30 min. The expression of surface-associated CD69 was then analyzed by flow cytometry using a Beckman flow cytometer.

Signal transduction studies

For analysis of the phosphorylation status of ZAP70, $\sim 3 \times 10^6$ cells were stimulated under the denoted culture conditions for the designated times. The stimulation was stopped immediately by chilling the cells on an ice water bath. Cells were then washed and lysed in 1 ml of 1× RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1% sodium deoxycholate, 1 mM sodium fluoride, 1 mM Na_3VO_4, 1 mM DTT, 1 mM PMSF, 1× protease inhibitor cocktail (Boehringer Mannheim, IN), and 1 μM microcystein-LR (CalBiochem, La Jolla, CA)). Equal amounts of cell lysates were separated on a 4–15% gradient SDS-PAGE gel. After electroblotting, the blot was probed with an anti-ZAP70 mAb (BD Transduction Laboratories) or a phosphotyrosine-specific mAb, RC 20 (BD Transduction Laboratories), and protein levels were detected by enhanced chemiluminescence detection reagents (Pierce, Rockford, IL).

NF-AT transactivation chloramphenicol acetyltransferase (CAT) assay

Overnight cultures of Jurkat cells were resuspended in sterile, ice-cold PBS and transfected with NF-AT CAT reporter construct by electroporation, as described earlier (22, 26). The transfected cells were diluted to 1 million cells/ml in prewarmed RPMI 1640 medium plus 0.1% BSA. Two milliliters of the cell suspension were distributed into each well of six-well culture plates with or without precoating with 1 μg of anti-CD3 Ab. TSP1 and other reagents in solution were added separately. After incubation at 37°C for 8 h, cells were isolated and washed extensively with 1× PBS. The cells were then lysed by five cycles of freeze and thaw treatment (10 min in dry ice, followed by 5 min at 37°C). CAT activity from the cell lysates was then measured as described previously (22, 26). The NF-AT transactivation activity under differential stimulation conditions was expressed as CAT activity quantified by PhosphorImager analysis (Molecular Dynamics).

Results

TSP1 inhibits the expression of early T cell activation marker genes

To globally define the biological responses of T cells to TSP1, we performed cDNA microarray analysis using glass slide microarrays containing about 2000 sequence verified human genes. We compared the changes in gene expression following TCR stimulation alone with that following TCR stimulation in the presence of TSP1. Less than 2% of the genes showed responses to TSP1, and these were both up-regulated and down-regulated. After stimulation with anti-CD3 for 4 h in a representative experiment, 1.7% (37/2200) of the genes showed increased expression, while 2.4% (53/2200) of the genes showed decreased gene expression. In the presence of 45 μg/ml TSP1, 1% of the genes (22/2200) in CD3-stimulated T cells had increased expression, and 0.4% of the genes (8/2200) showed suppressed gene expression relative to an anti-CD3-stimulated control. Based on analysis of eight independent mRNA preparations, we identified several genes whose mRNA expression was consistently altered by TSP1.

Expression levels of CD69, early growth response gene-1 (Egr-1), phosphatase of activated cells (PAC-1), TIEG/Egr-α

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(TGF-β-inducible early gene), the cytoplasmic CD27-binding protein (Siva, CD27BP), Fas-activated serine/threonine kinase (FAST), and protein phosphatase 2A (PP2A) were consistently altered in cells treated with TSP1 (Fig. 1). In the absence of TSP1, anti-CD3-stimulated T cells showed a time-dependent increase in expression of CD69, Egr-1, PAC-1, and TIEG/Egrα mRNAs (Fig. 1*A*). Among these, CD69, Egr-1, and PAC-1 have been classified as early activation markers for T cell activation (27–31). Expression of the four genes was elevated after 1 h, peaked at 4 h, and declined substantially after 8 h. But in the presence of TSP1, the TCR-stimulated expression of these four genes was all strongly inhibited. Conversely, mRNAs for FAST, CD27BP, and PP2A showed reproducible decreases in expression following TCR stimulation, but exhibited increased expression in the presence of TSP1 (Fig. 1*B*). This suggested that TSP1 antagonizes both the positive and negative effects of TCR stimulation on Jurkat T cell gene expression.

To further study this response to TSP1, we used an RNase protection assay to examine expression of IL-2, the primary T cell growth factor that is induced following T cell activation. As shown in Fig. 1*C*, the increased expression of IL-2 in anti-CD3-activated T cells was strongly inhibited by TSP1 treatment, while the housekeeping genes GAPDH and L32 showed minimal changes in their expression. The inhibition by TSP1 was dose dependent, and the IL-2 expression induced by TCR stimulation was almost completely suppressed by addition of 62.5 μg/ml (140 nM) of TSP1.

TSP1 specifically inhibits TCR-mediated T cell activation

To confirm our observations from microarray and RNase protection experiments, we examined the effects of TSP1 on expression of IL-2 and CD69 at the protein level. As shown in Fig. 2*A*, an ELISA demonstrated that the level of IL-2 secretion induced by anti-CD3 stimulation was indeed inhibited by TSP1. It should be noted that the anti-CD3 stimulation alone induces a relatively low IL-2 expression level. This was consistent with previous observations that partially activated T cells, such as stimulated by anti-CD3 alone, secreted low levels of IL-2 (32).

We examined the surface expression of CD69 induced by anti-CD3 treatment in both Jurkat T cells and freshly isolated human PBMCs. CD69 expression was strongly inhibited by TSP1 (Fig. 2*B*). This inhibition was potent and TSP1 dose dependent. At a concentration of 5 μg/ml (10 nM) of TSP1, expression of CD69 induced by TCR stimulation was inhibited (data not shown). Because some biological effects of TSP1 are mediated by activation of latent TGF-β1 (19), we examined the role of TGF-β1 in the inhibitory activity of TSP1 in TCR signaling. The inhibition of CD69 expression was TGF-β1 independent, because a potent TGF-β1-neutralizing Ab did not affect the inhibitory effect of TSP1 at a concentration of the Ab sufficient to neutralize about 250 ng of activated TGF-β1 (data not shown). TSP1 specificity was further supported by the fact that this inhibition was dependent on two known TSP1 receptors (see below).

Both soluble and immobilized TSP1 inhibit T cell activation

A recent study using an autoreactive T cell clone isolated from rheumatoid arthritis synovium suggested that TSP1 enhances T cell activation (25). In this study, TSP1 was used in immobilized form and adsorbed from a buffer at pH 9. To test whether immobilized TSP1 also inhibits TCR signaling and whether a different conformation of TSP1 might be induced by using buffer with a higher pH, we immobilized TSP1 on plates in either Dulbecco's PBS (pH 7) or bicarbonate buffer (pH 9) and examined CD69 expression levels in T cells plated on these substrates. We found that TSP1 immobilized under physiological conditions (PBS, pH

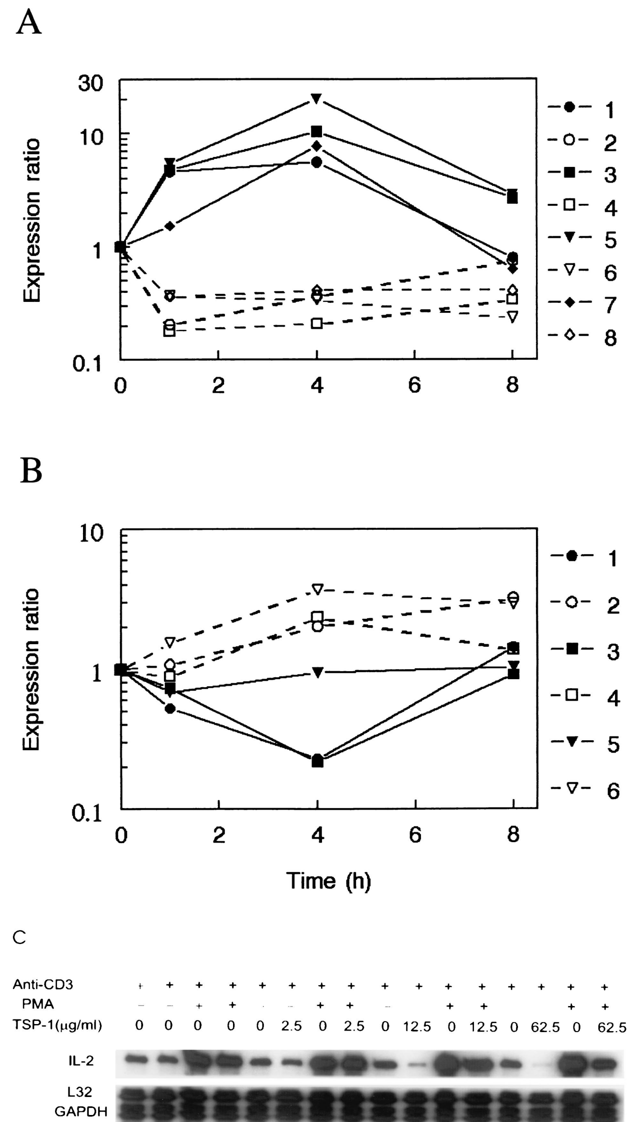

FIGURE 1. Microarray analysis revealed two sets of genes that were regulated by TCR and responded differentially to TSP1 treatment. *A*, Genes that were up-regulated after anti-CD3 stimulation, but were down-modulated by TSP1. CD69 expression after anti-CD3 stimulation (plot 1, ●) or plus TSP1 modulation (plot 2, ○); Egr-1 expression after anti-CD3 stimulation (plot 3, ■) or plus TSP1 modulation (plot 4, □); PAC-1 expression after anti-CD3 stimulation (plot 5, ▼) or plus TSP1 modulation (plot 6, ▽); and TIEG/Egr-α expression after anti-CD3 stimulation (plot 7, ◆) or after TSP1 modulation (plot 8, ◇). Plots with filled symbols and solid lines represent conditions only after anti-CD3 (1 μg/well) stimulation, while plots with open symbols and dotted lines represent conditions in the presence of TSP1 modulation (45 μg/ml). *B*, Genes that were down-regulated after anti-CD3 stimulation, but were up-modulated by TSP1. FAST expression with anti-CD3 stimulation (plot 1, ●) or plus TSP1 (plot 2, ○); CD27BP expression with anti-CD3 stimulation (plot 3, ■) or plus TSP1 (plot 4, □); and PP2A expression after anti-CD3 stimulation (plot 5, ▼) or plus TSP1 (plot 6, ▽). Plots with filled symbols and solid lines represent conditions with anti-CD3 stimulation, while plots with open symbols and dotted lines represent conditions plus TSP1 stimulation. Data were representative of eight different preparations of mRNA samples. *C*, RNase protection assay for IL-2 mRNA level. Jurkat T cells were stimulated with anti-CD3 Ab with or without 10 ng/ml PMA in the absence or presence of the indicated concentrations of TSP1 for 4 h. Total RNAs were isolated, and an RNase protection assay was used to quantify IL-2 mRNA levels. The housekeeping genes, GAPDH and L32, were used as loading controls. Data were representative of three independent experiments.

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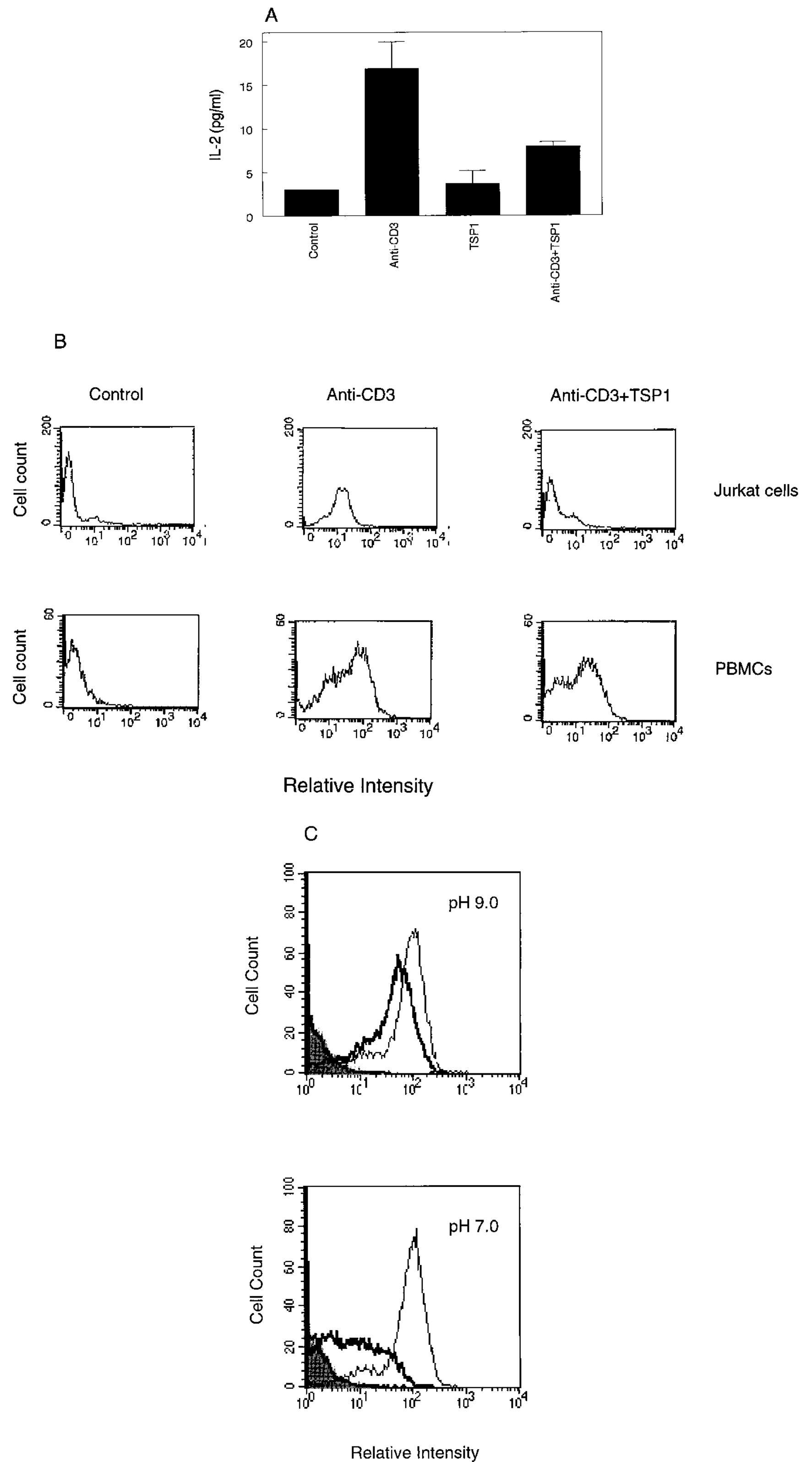

FIGURE 2. TSP1 inhibits IL-2 and CD69 protein expression on TCR-activated T cells. *A*, TSP1 effects on IL-2 secretion. Jurkat T cells were stimulated with or without precoated anti-CD3 Ab (1 μg/ml) plus or minus TSP1 (20 μg/ml). Culture supernatants collected after 24 h were used to measure IL-2 levels using the Quantikine ELISA kit. Data were representative of two different experiments. *B*, Effects of TSP1 on the surface CD69 expression. Jurkat T cells or freshly isolated human PBMCs were stimulated with or without surface-bound anti-CD3 Ab (1 μg/well) plus or minus TSP1 (30 μg/ml) added in solution. After incubation at 37°C for 20 h, cells were harvested and labeled with PE-conjugated anti-CD69 Ab (Coulter-Immunotech). Surface expression of CD69 was analyzed using a flow cytometer, as described in *Materials and Methods*. Starting from *left* to *right*, the CD69 expression of negative control, anti-CD3-stimulated T cells, and anti-CD3 plus TSP1-treated T cells are shown, respectively. The *upper panel* shows CD69 expression in the Jurkat T cells; the *lower panel* shows CD69 expression in PBMCs. Data were representative of three independent experiments (*C*). Effect of buffer pH on the activity of immobilized TSP1 to modulate surface CD69 expression. Jurkat T cells were cultured and stimulated as above. TSP1 was coimmobilized with anti-CD3 Ab on the plate surface in either carbonate buffer (pH 9) (*upper panel*) or Dulbecco's PBS (pH 7) (*lower panel*). Surface CD69 expression was measured by flow cytometry. Negative control (unstimulated) is shown as the shaded area. The thin-line curves represent anti-CD3-stimulated cells. The thick-line curves represent anti-CD3- and TSP1-treated cells. Data were representative of two different experiments.

7) inhibited anti-CD3-induced CD69 expression, but TSP1 immobilized at pH 9 lost most of its activity for inhibiting anti-CD3-stimulated CD69 expression (Fig. 2*C*). Thus, we concluded that both soluble and immobilized TSP1 are potent inhibitors for TCR-mediated T cell activation, but this inhibitory activity is lost when TSP1 is exposed to higher pH.

TSP1 inhibits TCR- but not PKC-mediated T cell stimulation

Although TSP1 could almost completely inhibit IL-2 gene expression in anti-CD3-stimulated T cells, it only partially inhibited the IL-2 gene expression induced in anti-CD3 plus PMA-stimulated T cells (Fig. 1*C*). The observation that TSP1 could only partially

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inhibit T cell activation induced by anti-CD3 plus PMA stimulation suggested that TSP1 might specifically antagonize the TCR signaling pathway. To test this hypothesis, we examined the inhibition by TSP1 of CD69 expression stimulated by a TCR-independent T cell activation pathway. As shown in Fig. 3*A*, TSP1 had no effect on CD69 expression in activated T cells mediated by ionomycin plus PMA stimulation. In addition, TSP1 showed no inhibitory activity on IL-2 expression in T cells stimulated by PMA plus anti-CD3 Ab (Fig. 3*B*). Similarly, we also found that TSP1 failed to inhibit CD69 expression in Con A-stimulated T cells (10 ng/ml) (data not shown). Additional experiments demonstrated that TSP1 did not inhibit CD69 expression in Jurkat T cells stimulated by suboptimal concentrations of PMA (0.5 ng/ml) and ionomycin (20 ng/ml), even though CD69 expression at this condition was lower than that of T cells stimulated by optimal concentration of PMA and ionomycin (data not shown). Furthermore, a higher concentration of TSP1 (90 μg/ml) could not inhibit CD69 expression in Jurkat T cells stimulated by PMA (10 ng/ml) and ionomycin (200 ng/ml) (data not shown). Taken together, our data suggested that TSP1 selectively inhibits anti-CD3-stimulated CD69 expression in Jurkat T cells.

TSP1 does not inhibit the upstream signaling pathway of TCR activation

To understand the molecular mechanism by which TSP1 selectively inhibits TCR-mediated T cell activation, we examined the effect of TSP1 on the TCR signaling pathway. ZAP70 is a critical upstream kinase in the TCR signaling pathway. TSP1 did not inhibit the phosphorylation of ZAP70 kinase induced by anti-CD3 stimulation (Fig. 4*A*). Consistent with these data, TSP1 did not inhibit the phosphorylation of linker for activation of T cells (LAT) (Fig. 4*A*) (22), an important adaptor protein in TCR signal transduction pathway that is a downstream target of ZAP70 (33). Subsequent examination showed equally expressed total ZAP70 and LAT in either stimulated or unstimulated samples with or without TSP1 treatment (data not shown). We therefore concluded that TSP1 did not exert an inhibitory effect through interference with the TCR upstream signal transduction pathway or by inhibiting anti-CD3 engaging its ligand.

To further confirm that TSP1 does not directly block anti-CD3-TCR interaction, we tested the time dependence for TSP1 to inhibit TCR-mediated T cell activation. TSP1 was added at 0, 15, or 60 min after T cells were stimulated by anti-CD3 Ab. CD69 expression was examined by flow cytometry. As shown in Fig. 4*B*, TSP1 could still inhibit T cell activation when added up to 60 min after the T cells were exposed to anti-CD3, while TSP1 has no effect on unstimulated cells (data not shown). This observation supported the conclusion that TSP1 inhibition is distal to early TCR upstream signal transduction.

The transcription factor NF-AT is an important downstream component of the signal transduction pathway in TCR-mediated T cell activation. Transactivation of NF-AT is induced by TCR-mediated T cell activation. As shown in Fig. 4*C*, TSP1 inhibited the transactivation of NF-AT induced by TCR engagement, indicating that NF-AT is one of the downstream targets involved in the inhibition by TSP1 of TCR-mediated T cell activation. Based on these data, we concluded that signals resulting from the interaction of T cells with TSP1 antagonized TCR signal transduction at a point upstream of NF-AT.

The inhibitory effect of TSP1 on T cell activation involves the TSP1 receptor CD47

We examined several cell surface TSP1 receptors that could mediate the inhibitory effect of TSP1. Among them, CD47/integrin-

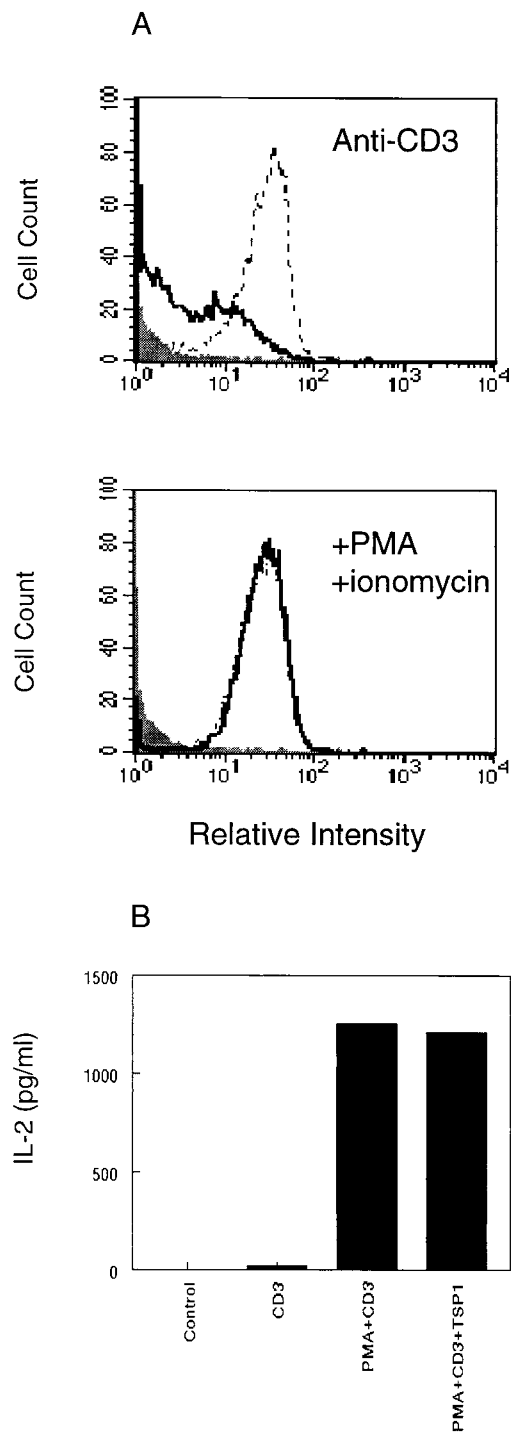

FIGURE 3. Effects of TSP1 on non-TCR-stimulated T cells. *A*, Effect of TSP1 on surface CD69 expression on either surface-bound anti-CD3 (1 μg/well)- or PMA (10 ng/ml) plus ionomycin (200 ng/ml)-stimulated T cells. Jurkat T cells were cultured and stimulated, and cell surface CD69 was stained, as described above. Negative controls (unstimulated cells) are shown as the shaded area. In the *upper panel*, the dotted-line curve represents anti-CD3-stimulated cells; the thick-line curve represents TSP1-treated cells with anti-CD3 stimulation. In the *lower panel*, the dotted-line curve represents PMA plus ionomycin-stimulated cells; the thick-line curve represents cells treated with TSP1 and PMA/ionomycin. Data were representative of two independent experiments. *B*, Effect of TSP1 on IL-2 expression in anti-CD3 (1 μg/well) plus PMA (10 ng/ml)-stimulated T cells. Jurkat T cells were cultured and stimulated as indicated. IL-2 levels in culture supernatants were quantified using the Quantikine ELISA kit, as described in *Materials and Methods*. Data were representative of two different experiments.

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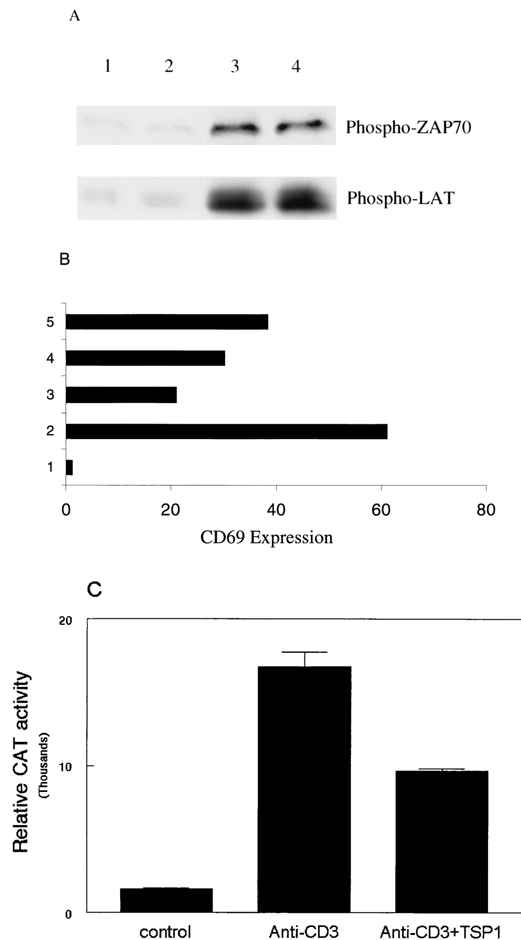

FIGURE 4. Effects of TSP1 on targets of the TCR signaling pathway. *A*, Western blot analysis of ZAP70 kinase. Overnight culture of Jurkat T cells was treated as following: unstimulated (*panel 1*), unstimulated cells plus TSP1 (30 μg/ml) (*panel 2*), stimulated with anti-CD3 (*panel 3*), or stimulated with anti-CD3 plus TSP1 (30 μg/ml) for 5 min at 37°C. The cells were immediately lysed in 1× RIPA buffer. Cell lysates were split into two parts, and equal amounts of protein were used for immunoprecipitation using either ZAP70- or LAT-specific antiserum (a generous gift from Dr. Samelson's laboratory, National Cancer Institute). The immunoprecipitated products were separated on a 4–15% gradient SDS-PAGE gel and blotted onto nitrocellulose membrane. Phosphorylated ZAP70 and LAT were detected by a phosphotyrosine-specific Ab (PY-20; Transduction Laboratories). *B*, Time dependence of TSP1 for inhibiting T cell activation. T cells were stimulated without (*panel 1*) or with (*panels 2–5*) precoated anti-CD3 Ab, as described earlier. TSP1 (30 μg/ml) was added at time 0 min (*panel 3*), 15 min (*panel 4*), and 60 min (*panel 5*) after anti-CD3 stimulation and continued to incubate at 37°C. Cells were stained for CD69 expression after incubation for 20 h. Flow cytometry analysis was used to measure surface CD69 expression. *C*, Effect of TSP1 on NF-AT transactivation in TCR-activated T cells. Jurkat T cells were transiently transfected with a NF-AT-CAT vector. The transfected T cells were pooled and then split evenly into three pools. Each pool was added into wells precoated with PBS, with 1 μg/well precoated anti-CD3 or with precoated anti-CD3 plus TSP1 in solution. The NF-AT transactivation activity represented by the CAT activity was measured as described earlier (22, 26) and is presented as mean ± SD. Data represent three different experiments.

associated protein has been recently shown to mediate an inhibitory effect of TSP1 on cytokine secretion in human monocytes (23) and dendritic cells (34). To examine whether CD47 plays a similar role in mediating the inhibitory effect of TSP1 on T cell activation, we examined the effect of a functional blocking CD47 Ab on the effect of TSP1 on T cell activation. Anti-CD47 Abs have been demonstrated with either agonist or antagonist activities for CD47 signaling (24, 35). We chose a well-established anti-CD47 mAb, B6H12, which blocked TSP1 binding to CD47 (36) and is a non-stimulating anti-CD47 Ab (35). As shown in Fig. 5*A*, preincubation of T cells with the CD47-blocking Ab partially reversed the inhibitory effect of TSP1 on T cell activation, demonstrating a specific blocking function resulting from the Ab preventing TSP1 binding to CD47.

We also examined the effect of a TSP1-derived CD47-binding peptide, peptide 7N3, on the expression of CD69 in TCR-stimulated T cells. As shown in Fig. 5*B*, the CD47-binding peptide 7N3 partially inhibited CD69 expression induced by TCR stimulation. The inhibition curve was peptide dose dependent, while a control peptide (604), which has two substituted amino acids in its CD47 binding site, had no effect on CD69 expression (Fig. 5*B*).

Some anti-CD47 Abs costimulate T cell activation and may mimic engagement of the CD47 counterreceptor signal-regulatory protein (37). Immobilized anti-CD47 (clone CIKm 1) did not enhance CD69 expression alone, but it strongly enhanced T cell activation mediated by TCR stimulation (Fig. 5*C*). To further elucidate the role of CD47 in the effect of TSP1 on T cells, we tested the effect of TSP1 on the costimulatory activity of immobilized anti-CD47. CD47 Ab CIKm 1 was coimmobilized with anti-CD3 Ab. As shown in Fig. 5*C*, TSP1 clearly antagonized the costimulatory effect of this CD47 Ab. The antagonist activity of TSP1 against CD47 costimulation was also TSP1 dose dependent.

Because this anti-CD47 is a costimulatory factor for IL-2 secretion in T cells, we also examined the effect of TSP1 on anti-CD47-stimulated IL-2 secretion (Fig. 5*D*). Coimmobilized anti-CD47 strongly costimulated TCR-mediated IL-2 secretion. Although TSP1 had no effect on T cells stimulated by anti-CD3 plus PMA (Fig. 3*B*), it inhibited IL-2 secretion stimulated by anti-CD3 plus anti-CD47.

Furthermore, we examined the downstream target NF-AT, which we showed to be involved in the inhibitory signaling pathway of TSP1, to see whether TSP1 antagonized costimulation by anti-CD47 of NF-AT transactivation activity (Fig. 5*E*). TSP1 antagonized the increased transactivation of NF-AT costimulated by anti-CD47 to a greater extent than it inhibited anti-CD3-induced NF-AT transactivation. These data support the conclusion that TSP1 specifically antagonizes the costimulatory effect of anti-CD47 Abs, and confirm a CD47 pathway dependency.

The inhibitory effect of TSP1 is also dependent on its heparin binding site

The CD47-blocking Ab could only partially block the inhibitory effects of TSP1 (Fig. 5*A*). This suggested that CD47 is not the only receptor mediating the inhibitory effects of TSP1. In fact, preliminary data using a CD47-deficient Jurkat cell line (38) showed that TSP1 could still inhibit anti-CD3-stimulated CD69 expression in cells lacking CD47 (data not shown).

To further explore potential TSP1 receptors that may mediate the inhibitory activity of TSP1, we screened a series of reagents that have been shown to antagonize specific biological functions of TSP1. Those included a TSP1-derived peptide that binds to CD36, heparin-binding peptides from the type I repeats, the focal adhesion-disrupting peptide Hep I, the RGD sequence, and several rTSP1 fragments. Among those we tested, only antagonists of the heparin binding site of TSP1 could reverse its inhibitory effect (Fig. 6 and data not shown). As shown in Fig. 6*A*, addition of heparin in solution almost completely abrogated the inhibitory effect of TSP1 on CD69 expression after TCR stimulation. Heparin could inhibit TSP1 binding to a T cell surface heparan sulfate

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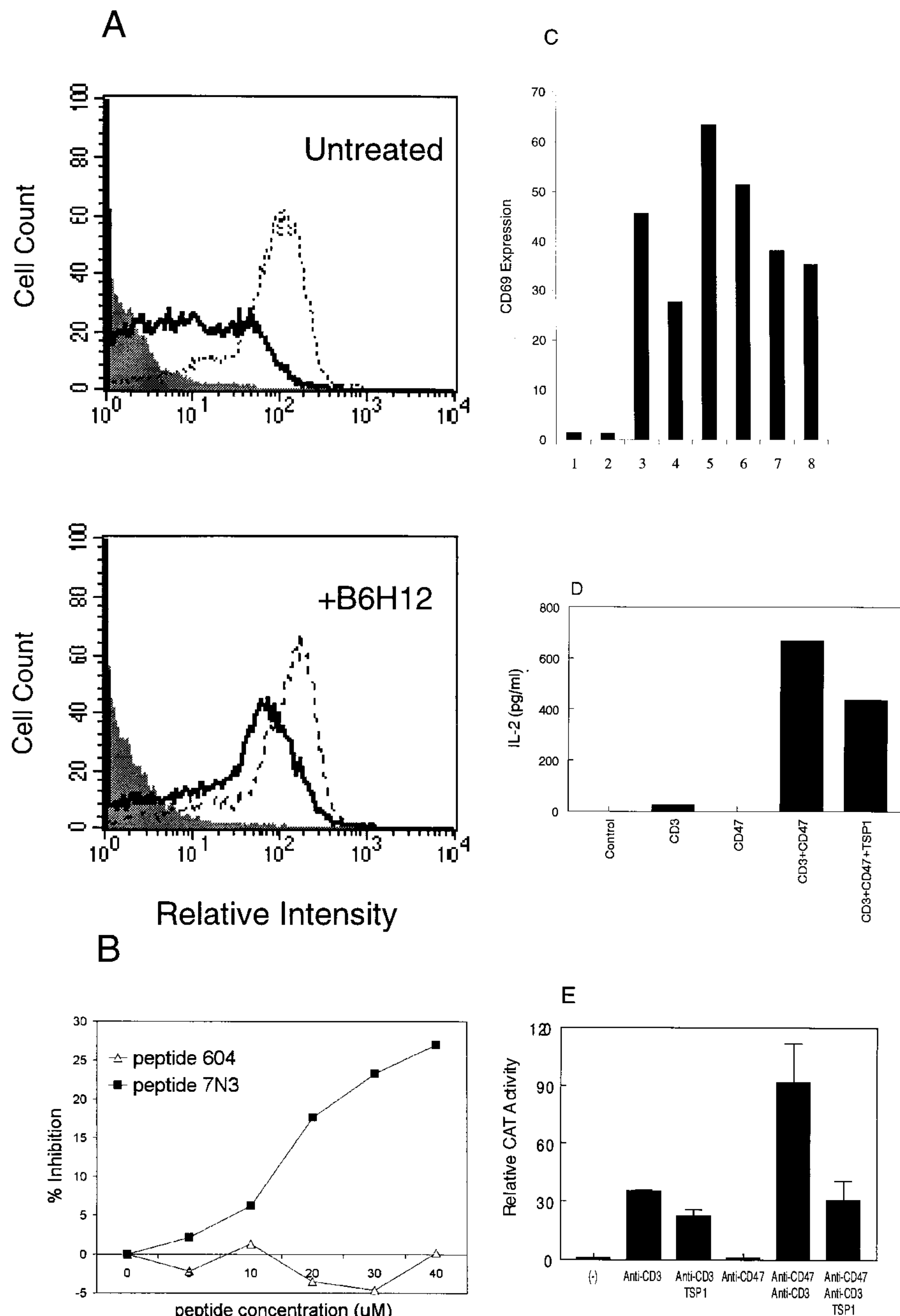

FIGURE 5. Involvement of the TSP1 receptor CD47 in the inhibitory activity of TSP1. *A*, A CD47-blocking Ab reverses the TSP1-inhibitory activity. T cells were stimulated with anti-CD3 Ab and treated with TSP1. A function-blocking CD47 Ab (B6H12; BD PharMingen) was used at 10 μg/ml to block TSP1 binding to the CD47 receptor. Surface CD69 expression was then analyzed using flow cytometry. Shaded areas represent negative controls. In the *upper panel*, the dotted-line curve represents anti-CD3 stimulation, while the thick-line curve represents anti-CD3 plus TSP1 treatment. In the *lower panel*, the dotted-line curve represents cells stimulated with anti-CD3 plus B6H12, while the thick-line curve represents anti-CD3 plus TSP1 plus the CD47-blocking Ab, B6H12. *B*, Effect of a CD47-binding peptide derived from TSP1. T cells were stimulated with or without surface-bound anti-CD3 Ab. A TSP1-derived CD47-binding peptide, 7N3, or the inactive control peptide 604 was added at the indicated concentrations. Surface CD69 expression of anti-CD3-activated T cells was measured by flow cytometry, as described earlier, and was normalized on the basis of that of nonstimulated T cells. The percentage inhibition of CD69 expression was calculated based on the CD69 expression on TCR-activated cells in the presence of either CD47-binding peptide (7N3) or its control mutant peptides (604) vs that of same anti-CD3-activated T cells in the absence of the peptides. The percentage of inhibition of CD69 was plotted against concentrations of each peptide. *C*, Effect of TSP1 on CD69 surface expression costimulated by anti-CD47 Ab. T cells were left unstimulated (*panel 1*), stimulated with precoated anti-CD47 (1 μg/well) (*panel 2*), anti-CD3 (1 μg/well) (*panel 3*), or anti-CD3 plus TSP1 (30 μg/well) (*panel 4*), or anti-CD3 (1 μg/well) coimmobilized with anti-CD47 (1 μg/well) without TSP1 (*panel 5*), or with different doses of TSP1 (5, 15, and 30 μg/well; *panels 6*, *7*, and *8*, respectively). The indicated concentrations of TSP1 were added, and surface CD69 expression was analyzed, as described earlier. Data represent one of three independent experiments (*D*). T cells were stimulated with immobilized anti-CD3 or anti-CD3 plus coimmobilized anti-CD47, as described in above. TSP1 was added to antagonize the costimulation of anti-CD47. IL-2 levels were quantified using an ELISA, as described above. Data were representative of two independent experiments. *E*, T cells were transiently transfected with a NF-AT-CAT vector, as described in Fig. 4*C*. Transfected cells were stimulated with anti-CD3 (1 μg/well) or anti-CD3 plus coimmobilized anti-CD47 (1 μg/well). TSP1 (30 μg/well) was added to antagonize the stimulation by anti-CD3 or anti-CD3 plus anti-CD47. CAT activity was measured as described earlier and is presented as the fold induction relative to an unstimulated control (mean ± SD). Data were representative of two independent experiments.

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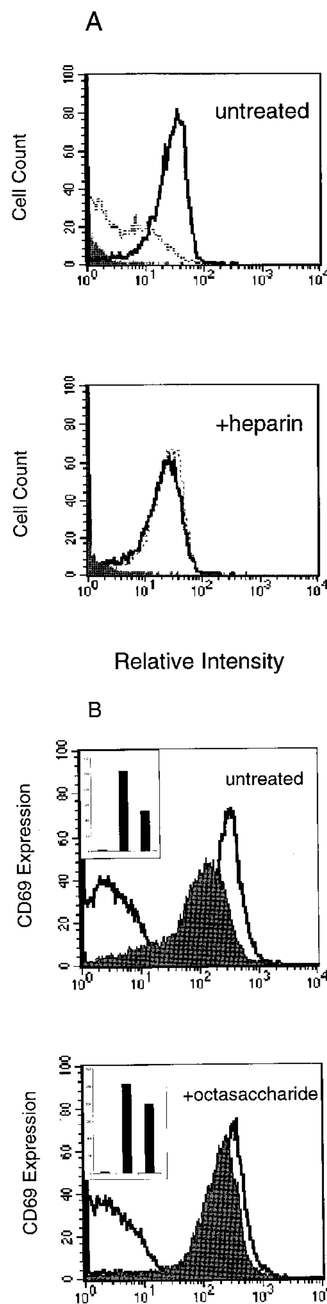

FIGURE 6. The heparin binding site of TSP1 contributes to its inhibitory activity. *A*, T cells were stimulated with surface-bound anti-CD3 (1 μg/well). TSP1 (30 μg/ml) was added to antagonize the TCR stimulation alone (*top panel*, untreated) or in the presence of heparin (10 μg/ml) (*lower panel*, +heparin) to prevent TSP1 binding to T cell HSPGs. CD69 expression was analyzed, as described earlier. Shaded areas are shown as negative controls for unstimulated cells. In the *upper panel*, the thick-line curve represents anti-CD3-stimulated cells; the dotted-line curve represents anti-CD3-stimulated cells plus TSP1 treatment. In the *lower panel*, the thick-line curve represents anti-CD3 stimulation plus heparin treatment, while the dotted-line curve represents anti-CD3 plus TSP1 plus heparin (10 μg/ml). Data were representative of two independent experiments (*B*). T cells were treated as in *A*. A heparin octasaccharide (0.5 mg/ml) that is monovalent for binding to TSP1 (*lower panel*, octasaccharide) was added to block the heparin binding sites of TSP1. CD69 expression was measured, as described earlier. In the *upper panel* (untreated), the shaded area represents anti-CD3 plus TSP1 treatment. In the *lower panel*, the shaded area represents anti-CD3 plus TSP1 plus octasaccharide treatment. The bar graphs in insets of each panel depict from *left* to *right* the relative CD69 expression level of unstimulated, anti-CD3-stimulated, and anti-CD3 plus TSP1-treated cells. Data were representative of two independent experiments.

proteoglycan (HSPG) receptor (22) that mediates an inhibitory signal. However, heparin is a large polysaccharide that could also physically sequester TSP1, by slowing the diffusion of the bound protein and inducing its aggregation, or sterically mask other functional sites on TSP1 that mediate the inhibitory signal. To distinguish between these mechanisms, we tested the effect of a heparin octasaccharide that is monovalent for binding to TSP1 (39) and is too small to physically sequester TSP1 or sterically block other functional sites on TSP1. As shown in Fig. 6*B*, this heparin oligosaccharide at a concentration 2-fold above its IC_{50} for inhibiting heparin binding to TSP1 (39) reversed the inhibitory effect of TSP1 on CD69 expression more than 2-fold. Therefore, the inhibitory effect of TSP1 on T cell activation mediated by TCR stimulation also requires its heparin binding sites.

Discussion

Although TSP1 is well known as an inhibitor of angiogenesis, and its tumor suppressor activity can be at least partly ascribed to inhibition of neovascularization, additional cell types may also be physiological targets of TSP1. In the immune system, TSP1 has known inhibitory activities in dendritic cells and thymic epithelial cells (34, 40). We have now demonstrated that TSP1 is a potent inhibitor of TCR-mediated T cell activation.

Based on a global microarray analysis, TSP1 reversed the changes in immediate-early gene expression induced by TCR-mediated T cell activation. One set of genes, CD69, Egr-1, TIEG, IL-2, and PAC-1, which were induced after TCR stimulation, was down-regulated by TSP1 treatment. mRNA expression levels for a second set of genes, PP2A, the cytoplasmic CD27BP Siva, and FAST, which were decreased after TCR stimulation, were up-regulated following TSP1 treatment. Furthermore, the inhibitory effects of TSP1 are specific for TCR-dependent gene expression. TSP1 strongly suppressed IL-2 expression in T cells stimulated with anti-CD3 alone, but showed much less inhibitory activity for PMA-stimulated T cells (Figs. 1*C*, 2*A*, and 3*B*). Similarly, TSP1 could inhibit anti-CD3-induced CD69 expression, but failed to inhibit CD69 expression induced by PMA plus ionomycin or by Con A.

Based on its described antiadhesive activities (17), we considered the possibility that TSP1 nonspecifically inhibits contact of T cells with the immobilized anti-CD3 used to trigger TCR signaling. Several results argue against this hypothesis. TSP1 inhibited CD69 expression even when added after the T cells was allowed to interact with immobilized anti-CD3 (Fig. 4*B*). Furthermore, upstream TCR signaling assessed by ZAP70 phosphorylation was not inhibited by TSP1 under the same conditions in which downstream signaling at NF-AT and immediate-early gene expression were suppressed. Therefore, we propose that TSP1 inhibits downstream TCR signaling through binding to specific TSP1 receptors and eliciting inhibitory signals that act downstream of the TCR.

It is notable that TSP1 induces reciprocal regulation of the phosphatases PP2A and PAC-1. PP2A is a well-known inhibitor of several kinase pathways involved in TCR signaling (reviewed in Ref. 41), so its up-regulation by TSP1 may inhibit T cell activation. PAC-1 induction during T cell activation has been described, but its function is unknown. Further study of the responses of these two regulatory phosphatases to TSP1 may clarify the signaling network that suppresses downstream TCR signaling despite transient activation of upstream Ras and MAP kinase signaling by TSP1 (22).

CD47 was first identified as an integrin-associated protein (42) that modulates signal transduction through several integrins (43–45) and was shown to be a TSP1 receptor (36). Studies in CD47-deficient transgenic mice demonstrated a pivotal role of CD47 in

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host immune responses to clear pathogenic bacterial infections (46), possibly due to its requirement for neutrophil extravasation (47). CD47 ligation also inhibits dendritic cell maturation (34) and synthesis of IL-12 in monocytes (23). In T cells, CD47 ligation enhances TCR-mediated T cell activation (24, 48) (Figs. 4 and 5). Although the costimulatory activity of immobilized CD47 Abs and a recent study using an autoreactive T cell clone from rheumatoid arthritis synovium (25) suggest that TSP1 would costimulate T cell activation by binding to this receptor, our present results demonstrate that the intact TSP1 molecule, either in solution or immobilized at a physiological pH, is a potent inhibitor for TCR-mediated T cell activation. We have shown both reversal of TSP1 inhibition of TCR signaling by an Ab known to prevent TSP1 binding to CD47 and the ability of TSP1 to antagonize the costimulatory activity of a second CD47 Ab. Furthermore, a TSP1-derived CD47-binding peptide specifically inhibited TCR-stimulated CD69 expression. Thus, we conclude that the whole TSP1 molecule induces an inhibitory signal that is at least partially dependent on its binding to CD47.

Because different CD47 Abs have been reported to either costimulate or inhibit T cell activation, ligation of this receptor may be expected to elicit both stimulatory and inhibitory signals. The molecular mechanism for the differential responses among several CD47 Abs and to intact TSP1 vs a stimulatory CD47-binding peptide derived from TSP1 (22) remains unknown. The physical state in which TSP1 is presented may control its effects on T cell activation. Based on our data, we propose that intact TSP1, in both immobilized and soluble forms, is primarily an inhibitor for TCR signaling. However, a conformation change or loss of disulfide bonds, such as may occur when TSP1 is immobilized in a high pH buffer, may suppress this inhibitory signal and allow positive T cell responses to TSP1 (25). Because a signal-regulatory protein was recently identified as a CD47 counterreceptor (37), further studies will also be required to determine whether TSP1 can antagonize CD47 signals resulting from engagement of this counterreceptor during Ag presentation.

We also found that the heparin binding sites in TSP1 are required for its inhibitory effect on TCR signaling. The heparin binding sites of TSP1 participate in its antiangiogenic activity (49), inhibiting proliferation of melanoma (50) and endothelial cells (51), and promoting cell motility (51, 52). TSP1 binds to several cell surface HSPGs, and among these the syndecans are known to transduce signals following ligand binding (53). We recently showed that heparin-binding peptides from TSP1 activate MAP kinase in T cells, leading to AP1-dependent transcription, which was inhibited by the sulfation inhibitor chlorate or treatment with heparatinase (22). Therefore, it is clear that binding of TSP1 to as yet unidentified HSPGs can elicit signal transduction in T cells. However, the direct effects of binding to this TSP1 receptor appear to be costimulatory rather than inhibitory of TCR signaling. Thus, we are not sure whether TSP1 can transduce negative signals directly via binding to a T cell HSPG or whether this interaction is required to facilitate binding of TSP1 to other signaling receptors.

An analogous function for HSPGs has been shown in melanoma cells, in which both the CD47 and heparin-binding sequences in TSP1 synergized with the RGD sequence in the type 3 repeats of TSP1 to stimulate signaling through binding of the latter sequence to $\alpha_v\beta_3$ integrin (44). Therefore, the reversal of TSP1 function in TCR signaling we observed using antagonists of CD47 and HSPG binding may result from loss of a negative signal from a third as yet unidentified TSP1 receptor. Alternatively, simultaneous engagement of CD47 and HSPG receptors may reverse the responses evoked by each alone. An interesting corollary of this model is that exposure to proteases, such as in wounds and in the proximity of an invading cancer, could release proteolytic fragments of TSP1 that regain the costimulatory activities observed using TSP1 peptides (22) or alkaline-denatured TSP1 (25).

Several matrix proteins have been implicated as costimulatory factors for T cells, but only tenascin was known previously to act as a negative regulator (15). The molecular mechanism by which tenascin inhibits T cell activation is unknown. In this study, we identified TSP1 as a potent and specific inhibitor of TCR-mediated T cell activation. We propose that TSP1 is a negative regulator of T cell activation that limits immune responses at sites in which it is released due to platelet activation or in which its expression is induced, including in wounds and tumor stroma (17). Our studies suggest that the inhibitory activity of TSP1 is mediated by a novel mechanism that requires both its CD47 and HSPG receptors. The biological significance of the negative regulation of T cells by TSP1 remains undetermined, but characterization of this signaling pathway will provide guidance for understanding the function of TSP1 in T cell activation and host immune responses.

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