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Involvement of the SHP-1 Tyrosine Phosphatase in Regulation of T Cell Selection

Jinyi Zhang,* Ally-Khan Somani,* Darren Yuen,* Ye Yang,* Paul E. Love,† and Katherine A. Siminovitch*

The selection events shaping T cell development in the thymus represent the outcome of TCR-driven intracellular signaling cascades evoked by Ag receptor interaction with cognate ligand. In view of data indicating TCR-evoked thymocyte proliferation to be negatively modulated by the SHP-1 tyrosine phosphatase, a potential role for SHP-1 in regulating selection processes was investigated by analysis of T cell development in H-Y TCR transgenic mice rendered SHP-1 deficient by introduction of the viable motheaten mutation or a dominant negative SHP-1-encoding transgene. Characterization of thymocyte and peripheral T cell populations in H-Y TCR-viable motheaten mice revealed TCR-evoked proliferation as well as the positive and negative selection of H-Y-specific thymocytes to be enhanced in these mice, thus implicating SHP-1 in the negative regulation of each of these processes. T cell selection processes were also augmented in H-Y TCR mice carrying a transgene driving lymphoid-restricted expression of a catalytically inert, dominant-negative form of SHP-1. SHP-1-negative effects on thymocyte TCR signaling were not influenced by co-cross-linking of the CD28 costimulatory and/or CTLA-4 inhibitory receptors and appear, accordingly, to be realized independently of these comodulators. These observations indicate that SHP-1 raises the signaling threshold required for both positive and negative selection and reveal the inhibitory effects of SHP-1 on TCR signaling to be cell autonomous. The demonstrated capacity for SHP-1 to inhibit TCR-evoked proliferation and selection indicate SHP-1 modulatory effects on the magnitude of TCR-generated signal to be a key factor in determining the cellular consequences of TCR-ligand interaction. The Journal of Immunology, 1999, 163: 3012–3021.

Signal delivery through the TCR plays an integral role in driving intrathymic T cell development and interaction of the TCR with cognate peptide/MHC, triggering a cascade of biochemical events that evoke either cell survival and maturation (positive selection) or cell death (negative selection). These selection processes are now known to depend upon the affinity/avidity of TCR interaction with MHC/peptide complex with high affinity/avidity interactions inducing cell death and deletion, and lower affinity/avidity interactions promoting T cell differentiation (1–6). These cellular responses are also shaped by a myriad of intracellular signaling events that provide a framework for downstream propagation of the selecting signals evoked by TCR ligand occupancy.

In mature T cells, downstream delivery of TCR-stimulatory signal requires the initial activation of the Src family protein tyrosine kinases (PTKs); Lck and Fyn, with consequent tyrosine phosphorylation of the TCR CD3 and ζ subunits, recruitment of the ZAP-70 PTK, and the sequential activation of a spectrum of signaling effectors that transduce the signal to the nucleus (7, 8). Not surprisingly, many of these same molecules have been shown to play pivotal roles in coupling TCR engagement to the selection of immature thymocytes. Thus, for example, data derived largely through the use of genetically altered mice have revealed either substantive or prerequisite roles for ZAP-70 (9), Lck and Fyn (10–12), p21ras (13), and mitogen-activated protein (MAP) kinase (14–16) in transducing TCR selection signals through the cell. However, at present, the specific signaling circuitry that enables ligand occupancy of a particular TCR to propagate a positive vs negative selecting stimulus is unclear.

In addition to TCR connections with effectors that promote intracellular signal relay, signaling function of the TCR is also subject to attenuation and suppression by a variety of receptors as well as cytokos proteins (17–22). Notable among these is the SHP-1 tyrosine phosphatase, an SH2-domain containing cytokos protein tyrosine phosphatase (PTP), now recognized as playing a prominent role in the negative regulation of both B and T cell Ag receptor signaling (19, 23, 24). In thymocytes and T cells, SHP-1 has been shown to suppress TCR-evoked proliferation signal through interactions with and dephosphorylation of TCR components, the Lck and ZAP-70 PTKs, and other downstream signaling effectors involved in TCR signal delivery (20–22). SHP-1 inhibitory effects on TCR-directed mitogenesis suggest that SHP-1 raises the threshold for TCR transmission of activation signals. Thus, this PTP is likely to act in concert with other TCR-signaling modulators to modify the strength of TCR signal and the biological outcome of TCR engagement. From this perspective, it appears likely that SHP-1 inhibitory effects on TCR signaling also translate to the modulation of thymocyte selection. To investigate this possibility, we have derived SHP-1-deficient mice expressing a male Ag H-Y-specific TCR transgene and analyzed T cell development and function in these animals. The results of this study reveal SHP-1 deficiency to be associated with increases in both positive and...
negative selection of H-Y-specific T cells. The data also indicate that SHP-1 effects on TCR-evoked thymocyte activation are real-ized independently of CD28 costimulatory or CTLA-4 inhibitory signals. These findings identify an integral role for SHP-1 in regulating thymocyte selection and demonstrate sensitivity of both the positive and negative selection processes to shifts in the magnitude of TCR signal.

Materials and Methods

Mice

Mice homozygous for the viable motheaten mutation (me<sup>+</sup>) were obtained by mating C57BL/6<sup>j</sup> me<sup>+</sup>/me<sup>+</sup> breeding pairs derived from the murine CDS coding sequence for the TCR<sub>ζ</sub> cDNA sequent in the construct <i>ζ</i>-CT108 (26). Founder lines were identified by Southern blotting, screened for expression of CDS by Northern blotting and flow cytometric analysis, and the mice were then backcrossed to C57BL/6<sup>j</sup> through six generations. These mice were then mated with H-Y TCR transgenic mice to generate H-Y TCR<sup>+/+</sup> mice. For derivation of CDS transgenic mice, a human CD2-CDS transgene was derived as previously detailed by substituting the murine CDS coding sequence for the TCR<sub>ζ</sub> cDNA sequent in the construct <i>ζ</i>-CT108 (26). Founder lines were identified by Southern blotting, screened for expression of CDS by Northern blotting and flow cytometric analysis, and the mice were then backcrossed to C57BL/6<sup>j</sup> through six generations. These mice were then mated with H-Y TCR transgenic mice to generate H-Y TCR/CD5<sup>+/+</sup> transgenics. To derive H-Y TCR/CD5<sup>+/+</sup> mice, the H-Y TCR/CD5 transgenics were mated to me<sup>+</sup>/me<sup>+</sup> mice and the F<sub>1</sub> H-Y TCR/CD5 transgenics were mated to me<sup>+</sup>/me<sup>+</sup> mice. The mice were typed for expression of the H-Y TCR and CDS transgenes using PCR amplification with the primer pairs 5′-CA GACCTCCTTGGATCTGCAGCCTAGT-3′ (forward) and 5′-CCGAGATCTGGGCGGCA AGAACCTCG-3′ (reverse) (27). The PCR amplification cycle (15 s at 94°C, 20 s at 64°C, and 30 s at 72°C) was repeated 35 times. H-Y TCR and CDS transgene expressions were also confirmed by staining of PBLs with PE-conjugated anti-Thy-1.2 and FITC-conjugated anti-V<sub>β</sub>8 Abs or FITC-conjugated anti-CD5 Abs, respectively. Mice were studied at the ages of 2–3 wk.

Generation of mice expressing a dominant-negative SHP-1 (dnSHP-1) transgene

The dnSHP-1 transgene was constructed by ligation of a SHP-1 cDNA containing a serine substitution of the cysteine residue at position 453 (28) into the BamHI site of the plLIT2 vector (29) downstream of a fragment containing the TCRβ promoter (1.7 kbp), the Igα enhancer (850 bp), and a 2-kbp sequence upstream of the <i>lek</i> gene promoter and upstream of coding sequences for the human growth hormone (hGH) gene. Dominant-negative activity of the SHP-1 Cys<sup>453</sup>→Ser mutant protein has previously been demonstrated by the enhanced phosphorylation of SHP-1 substrates in dnSHP-1-expressing cells (28). The 8-kbp HincII fragment (shown in Fig. 5A) was purified and microinjected at a concentration of 5 µg/ml into CD1 zygote pronuclei. Transgene-bearing founders were detected by Southern analysis of tail DNA using a 2.1-kb hGH gene probe and transgene expression was evaluated by Northern blotting of 10 µg total lymphoid tissue RNA using the hGH gene or SHP-1 cDNA probes to detect the transgene dnSHP-1 and endogenous SHP-1 transcripts and by immunoblotting analysis of lymphoid tissue cellular lysates using anti-SHP-1 and anti-SHP-2 Abs (see below). Founder mice were bred to the C57BL/6 background by backcrossing over six generations and were then mated with H-Y TCR transgenic mice to obtain H-Y TCR/dnSHP-1 double-transgenic animals.

Reagents

Abs used for these studies include FITC-conjugated anti-Thy-1.2, anti-CD8, and anti-CD5 Abs, PE-conjugated anti-CD4 and anti-CD4<sub>D</sub> Abs, and biotinylated anti-CD25, anti-CD25, anti-CD3, anti-TCR<sub>β</sub> Abs, and anti-V<sub>β</sub>8 Abs all from Pharmingen (La Jolla, CA). Biotinylated Ab to the H-Y specific TCR Var3 chain (T3.70) was generously provided by Dr. M. Julius (University Health Network, Ontario, Canada), and Cy5-conjugated streptavidin was purchased from Pharmingen. Monoclonal hamster anti-CD3 Ab 145-2C11 hybridoma (produced by Dr. R. Miller, Ontario Cancer Institute, Ontario, Canada) and purified from the supernatant by protein G chromatography. Hamster anti-mouse CTLA-4 Ab was a gift from Dr. L. Zhang (University Health Network, Ontario, Canada) and hamster anti-mouse CD28 Ab was purchased from Pharmingen. Rabbit polyclonal Ab to Erk-2 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), rabbit anti-hamster and anti-mouse IgG were from Jackson ImmunoResearch (West Grove, PA), and monoclonal anti-β actin Ab was from Sigma (St. Louis, MO). Rabbit polyclonal anti-SHP-1 Ab was produced in our laboratory (30) and rabbit polyclonal anti-SHP-2 Ab was generously provided by Dr. G-S. Feng (see Ref. 31). Chemicals used for immunoblotting/immunoprecipitation were purchased from Sigma.

Proliferation analysis

Single cell suspensions prepared from thymus, lymph nodes, or splenic tissues were subjected to erythrocyte lysis in ammonium chloride. For lymph node cell suspensions, membrane Ig<sup>+</sup> cells and macrophages were depleted by repetitive panning on rabbit anti-mouse IgG-coated tissue culture plates. T cells were further purified by negative affinity selection using T cell enrichment columns (R&D Systems, Minneapolis, MN). Thymocytes were then cultured for 72 h in 96-well flat-bottom microtiter plates (2 × 10<sup>6</sup> cells/ml) in culture medium alone (RPMI 1640 containing 10% heat-inactivated FCS, 50 µM 2-ME, and penicillin/streptomycin), or in the presence of varying concentrations of anti-CD3e (31–1250 ng/ml), anti-CD28 (0.5–5 µg/ml) or anti-CTLA-4 (0–10 µg/ml) Ab and 25 IU/ml IL-2; Sigma). Secondary rabbit anti-hamster IgG (4–5 µg/ml) was then added to cross-link the primary Ab. Mixed lymphocyte reactions were performed by 72-h coculture of lymph node T cells (2 × 10<sup>5</sup> cells/well) from female and male H-Y TCR, H-Y TCR/me<sup>+</sup> heterozygote, or H-Y TCR/me<sup>+</sup> homozygote mice with irradiated (3000 rad) splenocytes (5 × 10<sup>5</sup> cells/well) from male or female syngeneic (C57BL/6<sup>j</sup>) mice. Cultures were pulsed with [<sup>3</sup>H]thymidine (1 µCi/well, Dupont/New England Nuclear, Boston, MA) at 16 h prior to culture termination and the incorporated radioactivity was measured using an automated β scintillation counter.

Cell sorting and flow cytometric analysis

Cells (1–2 × 10<sup>5</sup>/sample) were resuspended in 100 µl immunofluorescence-staining buffer (PBS/1% BSA/0.05% sodium azide) and incubated with the appropriate fluorochrome-conjugated Abs for 30 min at 4°C. Biotinylated Abs used for tricolor staining were visualized using Cy5-conjugated Streptavidin. Stained cells were analyzed using a FACScan flow cytometer with CellQuest software (Becton Dickinson, San Diego, CA). For isolation of thymocyte subsets, 2 × 10<sup>5</sup> thymocytes from wild-type mice were stained with FITC-conjugated anti-CD8 and PE-conjugated anti-CD4 Abs and then subjected to cell sorting using a FACStarplus.

Immunoblotting analysis

Freshly isolated thymocytes (10<sup>7</sup>) from 3-wk-old wild-type and dnSHP-1 transgenic mice as well as 10<sup>5</sup> double-negative, double-positive CD4<sup>+</sup>CD<sup>8</sup> and CD4<sup>+</sup>CD<sup>8</sup> single-positive thymocytes obtained by sorting thymocytes from wild-type mice were resuspended in cold lysis buffer (1% Nonidet P-40, 50 mM HEPES (pH 7.2), 150 mM NaCl, 50 mM NaF, 50 mM phosphate, 50 mM ZnCl<sub>2</sub>, 2 mM EDTA, 2 mM sodium orthovanadate, and 2 mM PMSF) and the nuclei and unpulsed cells were then removed by centrifugation at 14,000 × g for 10 min at 4°C. Following evaluation of protein concentration, by the bicinchoninic acid method (Pierce Biochemicals, Rockford, IL), the lysate proteins were resuspended in SDS buffer, boiled for 5 min, electrophoresed through 12% SDS-polyacrylamide and the separated proteins then transferred to nitrocellulose (Schleicher & Schuell, Keene, NH). After 1-h incubation in TBST (150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 0.1% Tween-20, 0.1% gelatin) the filters were incubated with anti-SHP-1 Ab for 2 h at room temperature followed by goat anti-mouse antiserum labeled with peroxidase (Amersham, Arlington Heights, IL) and HRP conjugate (Bio-Rad, Hercules, CA) and visualized using an enhanced chemiluminescence system (Amersham). Filters were then restriped as per Amersham protocol and reprobed with anti-actin or anti-SHP-2 Ab.

Results

SHP-1 inhibits anti-CD3-induced thymocyte proliferation, but does not affect CD28 costimulatory or CTLA inhibitory signals

To begin exploring the influence of SHP-1 on T cell selection processes, two key modulators of TCR-driven T cell activation, the costimulatory CD28 (32) and negative regulatory CTLA-4 receptors (18), were investigated with respect to their potential relevance to SHP-1-mediated inhibition of TCR signaling. To this end, SHP-1-deficient thymocytes from mice homozygous for the viable

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motheaten (me<sup>e</sup>) mutation were used to assay the effects of CD28 and CTLA-4 cross-linking on TCR-induced proliferation. As is consistent with previous findings, the results of this analysis revealed me<sup>e</sup> thymocytes, cells which express a catalytically inert form of SHP-1 (30), to manifest enhanced proliferation relative to wild-type thymocytes in response to all doses of anti-CD3 Ab used for cell stimulation (Fig. 1A). Proliferation was also enhanced in the me<sup>e</sup> relative to wild-type cells following their co-stimulation with anti-CD3 and anti-CD28 Abs (Fig. 1B); augmented responses of the me<sup>e</sup> cells were again observed at all levels of anti-CD3 Ab stimulation (data not shown). However, a comparison of the anti-CD3 with anti-CD3/anti-CD28 proliferative responses of these cells revealed the extent that proliferation was increased in the me<sup>e</sup> relative to wild-type cells to be similar in both stimulatory contexts. Similarly, cross-linking of the CTLA-4 receptor engendered a reduction in proliferative responses of the SHP-1-deficient and wild-type cells that was proportionate to the levels of mitogenesis induced by anti-CD3/anti-CD28 co-stimulation. Thus, SHP-1 effects on TCR-elicited proliferation appear to be realized independently of the CD28 costimulatory and CTLA-4 negative regulatory receptors.

**Positive selection of H-Y-specific T cells is increased in H-Y TCR viable motheaten mice**

Although me<sup>e</sup> and me<sup>e</sup> mice manifest premature thymic involution, at 2–3 wk of age their thymi appear normal in terms of proportions of CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP) and CD4<sup>+</sup>CD8<sup>+</sup> single-positive (SP) cells (20). Similarly, expression of developmental and activation markers such as TCRαβ, CD25, CD44, CD69, and CD5 is also normal in these animals (data not shown). By contrast, thymic cellularity is somewhat decreased in the SHP-1-deficient mice, the total number of thymic cells in 2- to 3-wk-old me<sup>e</sup> mice being approximately 20% less than that observed in age-matched wild-type animals (data not shown). This observation, together with the capacity of SHP-1 to modulate TCR-induced proliferation, suggests that SHP-1 may also be relevant to thymocyte selection. To address this possibility, the viable motheaten mutation was bred into mice transgenic for the H-Y male Ag-specific TCR and the H-Y TCR/me<sup>e</sup> heterozygous and homozygous animals so derived then evaluated with respect to their thymic and peripheral T cell populations.

To determine whether expression of the H-Y TCR transgene modifies SHP-1 effects on TCR signaling, lymph node T cells from male and female H-Y TCR/me<sup>e</sup> mice were subjected to mixed lymphocyte reaction by coculture with splenocytes from syngeneic male or female mice. As shown in Fig. 2, both H-Y TCR/SHP-1-deficient and H-Y TCR cells responded to stimulation with male, but not female, splenocytes. However, H-Y specific mitogenesis was markedly enhanced in the H-Y TCR/me<sup>e</sup> relative to H-Y TCR T cells, the augmentation of proliferation in the SHP-1-deficient mice being comparable with that observed in me<sup>e</sup> relative to wild-type mice. Anti-CD3 Ab-induced increases in MAP kinase activation were also found to be augmented in thymocytes from H-Y TCR/SHP-1-deficient mice compared with H-Y TCR mice at 10 min after TCR stimulation (data not shown), a finding consistent with previous reports of enhanced MAP kinase activation in TCR stimulated me<sup>e</sup> thymocytes (20). Together, these data confirm the association of SHP-1 deficiency with enhanced TCR signaling and
imply that the impact of SHP-1 deficiency on TCR signal relay is not affected by introduction of the H-Y TCR transgene.

Previous data revealing SHP-1 expression to be down-regulated at the stage of germinal centroblast expansion (33) raise the possibility that SHP-1 levels also change during thymocyte development. To address this possibility, SHP-1 expression in various thymocyte subpopulations was examined by immunoblotting analysis of lysates from double-negative, DP, CD4CD8 SP and CD4 CD8 SP thymocytes purified by cell sorting. As shown in Fig. 3, the results of this analysis revealed SHP-1 levels to be comparable within each of these subpopulations. Thus, modulation of SHP-1 expression does not appear relevant to the roles this PTP plays at different stages of thymocyte ontogeny.

To evaluate the effects of SHP-1 on positive selection, thymocytes from female H-Y TCR and H-Y TCR/SHP-1-deficient mice were compared with respect to their CD4/CD8 and T3.70-staining profiles. The H-Y TCR, which can be detected by staining with either anti-clonotypic monoclonal T3.70 (for H-Y TCR Vα3 chain) or anti-Vβ8 Abs (for H-Y TCR Vβ8.2 chain), recognizes H-Y-related peptide presented on H-2Dα (3, 25). As is consistent with previous findings using this TCR transgene system (3, 25), the CD4 CD8 SP (CD8SP) thymocyte population is markedly expanded in H-Y TCR compared with wild-type female mice. A substantive portion of the CD8 SP thymocytes in these mice, however, manifest only modest expression of transgenic TCR α-chain (Fig. 4A), a phenomenon previously attributed to rearrangement and expression of the endogenous TCR α-chain genes during the DP stage of development (34). By comparison, the numbers of CD8 SP thymocytes showing TCRαβ expression is significantly increased in H-Y TCR female mice heterozygous for the me3 mutation (Fig. 4B), animals in which thymocytes express both wild-type and a mutant, catalytically inert, form of SHP-1 (30). Similarly, the CD8SP population was increased in size, and the ratio of CD8 SP/CD8 SP cells was also higher in H-Y TCR/me3 heterozygote (CD8 SP/CD8 SP ratio = 1.8) compared with H-Y TCR (CD8 SP/CD8 SP ratio = 0.9) thymuses. Increases in the proportions of CD8SP relative to CD4SP cells, as well as H-Y TCR αβ CD8SP cells, were even further exaggerated in female H-Y TCR/me3 homozygous mice (CD8 SP/CD8 SP ratio = 3.8) (Fig. 4A). Together, these observations indicate that positive selection is enhanced in the context of SHP-1 deficiency. This conclusion is also supported by data derived from analysis of peripheral T cell populations in female H-Y TCR/me3 mice. As illustrated in Fig. 4C, both the total CD8SP population and percentages of CD8 T cells expressing high levels of the transgenic H-Y TCR were found to be markedly enhanced in the H-Y TCR/me3 heterozygote, and even more so in the H-Y TCR/me3 homozgygous relative to wild-type H-Y TCR mice. Similarly, an analysis of total lymph node T...
cell populations revealed the numbers of cells showing high level expression of H-Y TCR α- and β-chains to be substantively higher in H-Y TCRmeα heterozygote and homozygote mice than in H-Y TCR females (Fig. 4D). Thus, the characteristics of the peripheral as well as thymic T cell populations studied here reveal SHP-1 deficiency to be associated with an enhancement in the positive selection of CD8hi H-Y TCR cells.

Positive selection is increased in H-Y TCR mice expressing a dnSHP-1 transgene

SHP-1 has previously been shown to attenuate activities of two PTKs (Lck and ZAP-70) pivotal to TCR-initiated signal transduction (21, 22). Accordingly, the alterations in TCR-driven selection processes detected in meα mice are likely to reflect T cell autonomous defects and not the secondary consequences of the multiple hemopoietic defects present in these mutant animals. However, to circumvent the latter situation, animals in which SHP-1 function was selectively reduced in lymphocyte lineages were derived using a transgene construct containing a cDNA encoding a dominant-negative form of SHP-1 (in which the cysteine residue at position 453 is replaced with a serine) under transcriptional control of the TCR Vβ promoter and Igμ enhancer sequences (Fig. 5A). This combination of promoter/enhancer elements has been shown to engender lymphoid-specific transgene expression detectable at the earliest stages of lymphopoiesis (29, 35). As is consistent with these observations, none of five founders in which expression of the dnSHP-1 transgene was detected developed the myeloïd/monocyte expansion observed in meα mice and viability of the animals appeared normal (data not shown). By contrast, analysis of one transgenic line (JS25) derived by backcrossing to C57BL/6J, revealed an effect of the dnSHP-1 transgene on both thymocyte development and function. In this line, expression of the dnSHP-1 transgene was found to be about twofold higher than that of endogenous SHP-1 in thymocytes, but was not significantly altered in lymph node T cells (Fig. 5B). As illustrated in Fig. 5C, anti-CD3-induced proliferative responses of the dnSHP-1 transgene-expressing thymocytes were substantively higher than those of wild-type thymocytes at all anti-CD3 stimulatory doses. Again, proliferation data derived by co-cross-linking of the TCR with CD28 and CTLA-4 Abs revealed SHP-1 deficiency to have no effect on CD28/CTLA-4 modulation of TCR signaling (data not shown). These findings thus mirror the differences apparent between similarly-treated meα and wild-type thymocytes and are also consistent with previous data revealing the capacity of the Cys453Ser SHP-1 mutant protein to function in a dominant-negative fashion (21, 28). To determine whether expression of the dnSHP-1 transgene also influences thymocyte selection in a manner similar to the meα mutation, mice from the dnSHP-1 transgenic line were crossed to H-Y TCR transgenic mice and the progeny were then examined with respect to T cell development. Although thymic cellularity and development appeared normal in the dnSHP-1 transgenic animals, phenotypic analysis of female thymocytes from H-Y TCR/dnSHP-1 double transgenic mice revealed a marked increase in the proportion of CD8SP (Fig. 6A) and H-Y TCRαhi CD8SP (Fig. 6B) thymocytes, a result that again indicates positive selection to be enhanced in the context of reduced SHP-1 function. Taken together with the data showing the generation/expansion of H-Y TCR-specific CD8SP cells to be increased in viable motheaten mice, these findings indicate a pivotal role for SHP-1 in down-regulating the TCR signals that stimulate positive selection.

Negative selection is enhanced in H-Y TCR SHP-1-deficient mice

Although analysis of the SHP-1-deficient H-Y TCR female mice revealed positive selection of H-Y TCR-specific CD8SP cells to be increased in these animals, the data also revealed the proportions...
To further address the impact of SHP-1 on negative selection, the effects of SHP-1 deficiency on thymocyte development were also studied in male C57BL/6 mice double transgenic for the H-Y TCR and for CD5, a pan-T cell transmembrane glycoprotein known to negatively regulate TCR signal delivery (17). These latter animals, in which T cell-specific CD5 overexpression is driven by the CD2 promoter/enhancer, have previously been shown to manifest a decrease in the negative selection of transgenic TCR-expressing thymocytes (Paul Love, unpublished data). This inhibitory effect of CD5 overexpression on negative selection was again detected in the current study, as revealed by the increased representation of DP cells in the thymi of H-Y TCR/CD5 compared with H-Y TCR mice (Fig. 7C). By contrast, introduction of the me mutation into the H-Y TCR/CD5 transgenic mice again dramatically decreased the size of the DP population and appears to virtually nullify the effects of CD5 overexpression. In addition, as illustrated in Fig. 7D, the deletion of DP thymocytes was also substantively increased in H-Y TCR/dnSHP-1 transgenic relative to H-Y TCR male mice as evidenced by a reduction in the proportions of DN cells in the double-transgenic animals. The enhanced deletion of H-Y-specific thymocytes associated with expression of the dnSHP-1 transgene also impacted upon the profile of T cells found in the periphery, the proportions of CD8SP cells being significantly reduced in male H-Y TCR/dnSHP-1 double transgenic relative to H-Y TCR lymph nodes (Fig. 7D, lower panel). Taken together, these observations indicate that SHP-1 effects on T cell selection are realized cell autonomously and provide confirmatory evidence of the role for SHP-1 in suppressing TCR-directed negative as well as positive selection in the thymus.

**Discussion**

The biochemical mechanisms that translate TCR interaction with cognate peptide/MHC into the positive or negative selection of developing thymocytes are of key relevance to the understanding of tolerance acquisition and maintenance. In view of previous data revealing Ag-receptor driven mitogenesis to be aberrantly increased in thymocytes and mature T cells from SHP-1-deficient motheaten mice, we have used the H-Y TCR transgene system to investigate the possibility that SHP-1 influence on TCR signaling also translates to the regulation of intrathymic selection processes. The results of this analysis revealed TCR-induced proliferation to be augmented in both H-Y TCR/meme T cells, and thymocytes expressing a dsSHP-1 protein. SHP-1 inhibitory effects on TCR signaling were found to be unaltered by CD28 or CTLA-4 co-crosslinking and to dramatically influence T cell selection events in H-Y TCR transgenic mice. In H-Y TCR female mice, for example, SHP-1 deficiency conferred by either the me mutation or the dnSHP-1 protein was associated with marked increases in the H-Y TCR/meme thymic and peripheral T cell populations. SHP-1 deficiency also altered T cell development in H-Y TCR male mice, resulting in a marked reduction in the DP thymocyte and in CD8SP peripheral T cell populations. Taken together, these data indicate that SHP-1 effects on TCR signaling are cell autonomous, occur independently of CD28 and CTLA-4, and translate not only to the down-regulation of T cell proliferation, but also to the suppression of TCR-directed maturation/expansion and clonal deletion in the thymus.

In the current study, the expansion of CD8SP and H-Y TCR α- and β-chain-expressing T cell populations in SHP-1-deficient mice compared with control H-Y TCR female mice provides strong evidence of a role for SHP-1 in down-regulating the process of positive selection. Similarly, this role for SHP-1 was also revealed by
FIGURE 7. SHP-1 deficiency is associated with increases in the negative selection of H-Y-specific T cells in H-Y TCR transgenic mice. Thymocytes or peripheral lymph node T cells were obtained from male H-Y TCR wild-type (H-Y +/+), H-Y viable motheaten heterozygote (H-Y Me'/+), viable motheaten homozygote (H-Y Me'/Me'), CD5 transgenic (H-Y CD5), CD5 transgenic/viable motheaten (H-Y CD5 me'/me') and dnSHP-1 (H-Y dnSHP-1) mice. Cells were stained with anti-CD8-FITC and anti-CD4-PE and then subjected to flow cytometric analysis. Percentages of stained cells are shown in each quadrant or boxed area. Results are representative of five independent experiments. A, Two parameter histograms show CD4 and CD8 staining profiles in thymocytes from wild-type, me' heterozygote, and me' homozygote H-Y TCR male mice. B, Two parameter histograms showing CD4 and CD8 expression in lymph nodes from H-Y TCR (H-Y +/+ ) and H-Y TCR/me' (H-Y me'/me') mice. C, Two parameter histograms showing CD4 and CD8 staining profiles in thymocytes from wild-type, CD5 transgenic, and CD5 transgenic viable motheaten H-Y TCR male mice. D, Two parameter histograms showing CD4 and CD8 expression in thymocytes (upper panel) and lymph node T cells (lower panel) from wild-type and dnSHP-1 H-Y TCR male mice.
the finding that DP and lineage committed CD4<sup>low</sup> CD8<sup>+</sup> transitional thymocytes from the SHP-1-deficient H-Y TCR mice expressed higher levels of CD69 and Bcl-2 and were also morphologically more blastic than the comparable cells from control H-Y TCR females (data not shown). An inhibitory effect of SHP-1 on positive selection is consistent with previous data identifying SHP-1 as a negative modulator of TCR signaling and suggesting that this effect of SHP-1 may involve its down-regulation of Lck, ZAP-70, and MAP kinase activation (20–22). Importantly, while the intracellular circuitry transducing positive selecting signals is not well understood, ZAP-70, Lck, and MAP kinase activation have all been implicated in this facet of T cell development (9–11, 14, 15). Thus, it appears likely that modulation of these particular TCR-signaling components accounts, at least in part, for the capacity of SHP-1 to inhibit positive selection. However, SHP-1 also interacts with subunits of the TCR complex (20), the Vav protein (20, 36), and phosphatidylinositol 3-kinase (37), and the extent to which these latter interactions are also relevant to SHP-1 effects on selection remains to be determined.

An association of SHP-1 deficiency with enhanced negative selection was also detected in the current study, the data revealing numbers of DP thymocytes as well as CD8<sup>SP</sup> thymocytes and peripheral T cells to be reduced in SHP-1-deficient H-Y TCR male mice. Importantly, while total thymic cellularity has been found to be severely reduced in older (5- to 6-wk-old) me<sup>e</sup> homozygote mice, the decrease in total thymic cellularity in 2- to 3-wk-old me<sup>e</sup> mice is much less significant and unlikely to account for the reduction in thymocyte subpopulations detected in H-Y TCR/me<sup>e</sup> male mice. This contention is supported by the fact that the DP thymocyte population of H-Y TCR male mice is also reduced in conjunction with me<sup>e</sup> heterozygosity or expression of a dnSHP-1 transgene, situations in which total thymic cellularity remains intact. Thus, while altered thymocyte cellularity has previously been ascribed to impaired recruitment of thymocyte progenitors from the bone marrow (38) as well as the deleterious influence of over-expanded macrophage/myeloid populations (39), the data reported here indicate that increases in intrathymic negative selection also contribute to this facet of the thymothen phenotype. This conclusion is also consistent with the apparent capacity of SHP-1 deficiency to counteract the inhibitory effects of CD5 overexpression on negative selection, an observation that also raises the possibility that SHP-1 activity is required for CD5 to realize its inhibitory effects on negative selection. A role for SHP-1 in inhibiting the negative selection of immature thymocytes is also supported by data demonstrating SHP-1-deficient autoreactive B cell precursors in the marrow to manifest a heightened susceptibility to clonal deletion (40).

As illustrated in Fig. 4A, characterization of the thymocyte populations in SHP-1-deficient H-Y TCR female mice revealed the CD8<sup>+</sup>/CD4<sup>+</sup>SP ratio and the numbers of H-Y TCR<sup>α<sup>high</sup></sup> CD8<sup>+</sup> cells to be higher in the me<sup>e</sup> homozygous than in me<sup>e</sup> heterozygote H-Y TCR mice. By contrast, me<sup>e</sup> homozygosity was associated with a reduction in the DP, CD4<sup>SP</sup>, and CD8<sup>SP</sup> thymic populations. This finding cannot be ascribed to the overall reduction in thymic cellularity manifested by 2- to 3-wk-old me<sup>e</sup> mice as relative proportions of the major thymocyte subsets are not altered in these latter mice (20). Thus, the reduction of the DN, CD4<sup>SP</sup>, and CD8<sup>SP</sup> populations observed in the H-Y TCR me<sup>e</sup> homozygous mice implies the association of me<sup>e</sup> homozygosity with increased clonal deletion and the capacity of H-Y TCR T cells to undergo negative selection in the absence of H-Y Ag. As CD4<sup>SP</sup> cells are not susceptible to negative selection in female H-Y TCR mice (41), the current data revealing proportions of these cells to be reduced in H-Y TCR/me<sup>e</sup> mice suggest that the deletion processes regulated by SHP-1 may occur during the transitional stage from DP to SP thymocytes prior to commitment to the CD4 or CD8 lineage. Along similar lines, negative selection has been previously shown to occur in the absence of agonist peptide under conditions that promote thymocyte interactions with the stromal cell milieu, as exemplified by induced increases in CD8 expression (42, 43) or in the concentration or affinity of TCR-interacting peptide (1, 44).

Hemopoietic development has been extensively studied in me and me<sup>e</sup> mice and the results of such studies have linked at least some of the lymphoid cell defects observed in these animals to the overexpansion of myeloid/monocytic cell populations (45–47). This contention is consistent with recent data revealing the capacity of SHP-1 to negatively regulate myeloid cell proliferation and function (48), to down-regulate receptors such as the IL-3, macrophage CSF and granulocyte-macrophage receptors, which promote myelomonocytic growth and development, (49–51), and to interact with cell surface proteins such as PIR-B and SHPS-1, which are implicated in the negative regulation of macrophage growth (52–54). In view of these observations, the degree to which the T lymphoid defects observed in me and me<sup>e</sup> mice are intrinsic to T lineage cells was evaluated in the current study using mice in which a dominant-negative form of SHP-1 was selectively expressed in thymic T cells. Although these latter animals show no evidence of B cell or myelomonocytic abnormalities (data not shown), TCR-induced thymocyte proliferation as well as positive and negative selection processes were found to be markedly enhanced in these mice, the findings essentially mirroring those observed in me<sup>e</sup> mice. Thus, while the myeloid/monocytic expansion associated with the me and me<sup>e</sup> mutations likely impacts on some facets of lymphoid physiology, the current data provide compelling evidence that SHP-1 modulates T cell function in a cell autonomous fashion. This conclusion is also supported by previous data revealing TCR-evoked proliferation to be markedly augmented in Jurkat T cell transfectants expressing a dnSHP-1 protein (21), and also by multiple lines of evidence identifying SHP-1 as a modulator of signaling effects key to TCR signal relay (20–22).

Previous data concerning SHP-1 functions in B cells have revealed SHP-1 inhibitory effects on B cell Ag receptor signaling to be realized in part through interactions with BCR comodulators such as CD22, FcγRIIB, PIR-B, and CD72 (55–58). These data, together with previous findings implicating CD28 in the apoptotic processes that engender negative selection (59), prompted our investigation as to whether SHP-1 effects on T cell activation might involve modulation of the CD28 costimulatory and/or CTLA-4 negative regulatory receptors. The current data, however, revealing that CD28 and CTLA-4 effects on TCR-evoked proliferation are not modified by SHP-1 deficiency, indicate SHP-1 inhibitory influence on TCR signaling to be realized independently of these receptors. These results are consistent with the absence in both the CD28 and CTLA-4 cytosolic domains of the SHP-1 SH2 domain-interacting immunoreceptor tyrosine-based inhibition motifs. A lack of SHP-1 effect on CTLA-4 modulatory function is also supported by data demonstrating that CTLA-4 associates with SHP-2, but not SHP-1 (60), and that thymic selection proceeds normally in CTLA-4-deficient mice (27).

The data reported here indicate both positive and negative selection to be markedly influenced by the magnitude of signals emanating from the TCR and also reveal SHP-1-mediated increases in the threshold for TCR signal delivery to have multiple biological consequences. Taken together with previous findings from studies of ZAP-70 and TCR γ knockout mice (9, 61), the current data also suggest a commonality in the most proximal intracellular signaling events that regulate both positive and negative selection. Thus, the
translation of varying affinity/avidity TCR interactions with cognate ligand to the induction of these two different biological outcomes appears to be mediated by more downstream biochemical events, a conclusion also suggested by the selection defect associated with disruption of the MAP kinase cascade (14, 16). Similarly, the detection in DN thymocytes of SHP-1 levels comparable with those detected in DP and SP cells suggests that SHP-1 may also impact upon other facets of thymocyte ontogeny by modulating pre-TCR signaling (62). While this possibility as well as the mechanisms whereby SHP-1 modulates selection processes require further investigation, the data presented here suggest that SHP-1 effects on TCR signaling influence the transition between positive and negative selection and, by extension, the cellular events that engender autoimmunity.

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