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The tyrosine phosphatase SHP-1 influences thymocyte selection by setting TCR signaling thresholds

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Abstract

Modulation of the strength of signals from the TCR determines the outcome of positive and negative selection in thymocyte development. Previous studies have demonstrated that SHP-1 plays a role in determining signal strength from the TCR. Here, we have taken a genetic approach to test whether SHP-1 plays a role in T cell selection in the thymus. Experiments in which a dominant negative mutant of SHP-1 was expressed in the BYDP hybridoma cell line confirmed that SHP-1 regulated TCR signaling in a cell-autonomous manner and suggested that Lck is one of its targets. To examine the role of SHP-1 in T cell development, we crossed the ovalbumin-specific DO11.10 TCR transgene onto the *motheaten* background, which lacks SHP-1 expression. Analysis of the progeny of these crosses provided evidence that SHP-1 regulates thymocyte selection: (i) flow cytometric analyses revealed alterations in the percentages of thymocyte subpopulations in the *me/me* background; (ii) *ex vivo* deletion experiments demonstrated that *me/me*:Tg thymocytes undergo negative selection at lower concentrations of OVA peptide compared to +/+:Tg thymocytes; and (iii) *ex vivo* proliferation analyses indicated that *me/me*:Tg thymocytes were hyper-sensitive to stimulation by the specific OVA peptide. Our observation that the absence of SHP-1 leads to altered selection of TCR transgenic thymocytes demonstrates that SHP-1 regulates the strength of TCR-mediated signals *in vivo* and, in turn, helps to set the threshold for thymocyte selection.

Introduction

Tyrosyl phosphorylation and dephosphorylation of proteins comprise key regulatory events in many signal transduction pathways leading to proliferation and differentiation. In T cells, the TCR signals via the activation of protein tyrosine kinases (PTK). The TCR is composed of antigen-specific α and β chains along with several invariant chains of the CD3 complex, the γ, δ, ε and ζ chains (reviewed in 1,2). Upon antigen presentation by MHC molecules, the $\alpha\beta$ TCR binds to the antigen and intracellular signals are propagated via the cytoplasmic tails of the CD3 chains. MHC molecules also serve as ligands for the co-receptors CD4 and CD8 on T cells. These co-receptors (along with their associated PTK Lck) are concomitantly activated through their interaction with the same MHC molecule as the one recognized by the TCR. Upon receptor engagement with antigen, several CD3 chains, most notably the ζ chain, become heavily tyrosyl phosphorylated which, at least in part, is catalyzed by Lck. A similar effect can be evoked by antibody cross-linking of the receptor. The resulting phosphotyrosyl residues provide docking sites for recruitment of several SH2 domain-containing proteins, including the PTK Zap-70. The subsequent activation of Zap-70 leads to downstream tyrosyl phosphorylation of cellular proteins.

TCR-initiated signaling pathways play a critical role in determining thymocyte fate during development. The major developmental stages of immature T cells in the thymus are defined by the cell surface expression of the TCR and the co-receptors CD4 and CD8. Immature pre-thymocytes (which

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originate in the bone marrow) colonize the thymus and first differentiate into 'triple-negative' thymocytes that do not express the TCR, CD4 or CD8. These cells then acquire low levels of TCR expression without the expression of CD4 or CD8 [double-negative (DN)]. Via a $TCR^+CD4^-CD8^{low}$ transitional stage, DN thymocytes differentiate into a double-positive (DP) state, in which both CD4 and CD8 are co-expressed with the TCR. DP thymocytes undergo either positive selection (which leads to further maturation and proliferation of selected cells), culminating in the production of $CD4^+$ or $CD8^+$ single-positive (SP) cells, or negative selection (which leads to cell death). In addition to these major stages in T cell differentiation, a number of intermediate states of thymocyte development have also been defined based on the expression of other surface molecules (reviewed in 3,4).

The different stages of T cell maturation in the thymus have been elegantly studied in TCR transgenic mice (TCR Tg) carrying specific TCR α and β chains (reviewed in 5). The introduced TCR genes, which are expressed on almost all immature T cells, as well as on the majority of the mature T cells, provide a convenient approach for following the fate of thymocytes and mature T cells during development. Current models suggest that the TCR of an immature thymocyte interacts with a putative endogenous antigen (presented by thymic epithelial cells) and that the avidity of this T cell–antigen interaction determines the fate of the maturing thymocyte. Immature T cells with TCR that have a negligible or low avidity will die due to 'neglect'. Thymocytes that express TCR of moderate avidity will be positively selected for further maturation, while those expressing TCR of high avidity for the endogenous antigens will be negatively selected ('deleted') (6–8).

Mice with specific defects in signaling molecules have provided powerful tools to examine the function of these molecules in T cell development, in particular when crossed with TCR Tg mice. For example, mice with targeted disruption of the genes encoding Lck (9) or the tyrosine phosphatase CD45 (10), which regulates Lck, display blocks in thymocyte development, indicating the importance of these molecules as positive regulators of T cell maturation. Conversely, crosses between the TCR Tg and CD5-deficient mice identified CD5 as a negative regulator of thymocyte selection process (11).

Although much is known about the initiation of the TCR-mediated signaling events, very little is known about mechanism(s) by which TCR signaling is terminated. The tyrosine phosphatase SHP-1, which is expressed predominantly in hematopoietic cells, has been implicated in negative regulation of signaling events induced by receptors for cytokines, growth factors and antigens (12,13). SHP-1 is composed of a central catalytic domain, two Src-homology 2 (SH2) domains at its N-terminus and a C-terminus with tyrosyl phosphorylation sites (14). The best evidence for a critical role for SHP-1 *in vivo* has come from the *motheaten* mouse, a naturally existing genetic model for SHP-1 deficiency (15–17). Mice homozygotic for the *motheaten (me/me*) mutation at the SHP-1 locus express no detectable SHP-1 protein. These mice have proven to be a valuable tool for genetic studies to probe the *in vivo* functions of SHP-1 (18,19). The *me/me* mice display a multitude of disorders in all hematopoietic lineages. To date, no obvious phenotype has been reported for heterozygotic (*me*/+) mice.

We and others have previously implicated a role for SHP-1 in TCR-mediated signaling. In an earlier study, we showed that SHP-1 becomes rapidly tyrosyl phosphorylated upon cross-linking of the TCR (14). Recent studies (20,21) have demonstrated that, compared to thymocytes from normal mice, *me/me* thymocytes are hyper-responsive to TCR stimulation, which is manifested by increased IL-2 production and hyper-proliferation in response to TCR cross-linking. A similar hyper-sensitivity of TCR-mediated signaling was demonstrated by overexpression of dominant negative forms of SHP-1 in Jurkat T cells, which also resulted in enhanced IL-2 production (22). The precise target(s) of SHP-1 remain controversial. Plas *et al*. suggested Zap-70 as the direct target of SHP-1 (22). In contrast, our data showed that *me/me* thymocytes display a prolonged activation of Lck and Fyn kinases and increased intracellular tyrosyl phosphorylation of several substrates (20). Regardless of the direct target(s), the above data suggest that SHP-1 is a negative regulator of TCR-mediated signaling and that a lack of SHP-1 causes increased strength of TCR-mediated signals.

Since SHP-1 is implicated in negative regulation of TCR-mediated signaling and the strength of the signal transmitted from the TCR influences T cell maturation, we tested the hypothesis that regulation of TCR-mediated signals by SHP-1 influences thymocyte development by controlling the thresholds for positive and/or negative selection perceived by thymocytes. We hypothesized that a thymocyte expressing a TCR of low avidity, which, due to a weak TCR signal, would normally be 'neglected', in the absence of SHP-1, might signal with sufficient strength to be positively selected. Similarly, a thymocyte bearing a TCR of moderate avidity, which would normally be positively selected, might be deleted in the *me/me* background due to enhanced signals via the TCR. Thus, in the *me/me* mouse, the 'perceived' thresholds for thymic positive and negative selection would be 'leftward-shifted' with respect to antigen dose/avidity.

Although, we and others have failed to observe any abnormalities (as assessed by surface marker expression) in overall thymocyte composition in *me/me* mice (20,21,23,24), it is important to note that analyses of a *me/me* thymus may not show abnormalities in a steady state since the thymocyte selection process *per se* is not perturbed, but rather the TCR that are selected have different avidities. If SHP-1 controls signaling thresholds, the average positively selected *me/me* T cell might express a TCR with lower avidity than the average in a normal mouse. Such an effect would not easily be revealed in *me/me* mice expressing a large variety of unidentified TCR. However, a role for SHP-1 in thymocyte selection might be detectable if the majority of the thymocytes express a single TCR of a defined affinity (e.g. in a TCR transgenic mouse). Based on this rationale, we adopted a genetic approach, which allowed us to follow the fate of thymocytes in a controlled fashion by monitoring thymocyte populations in which the majority of cells express a specific Tg TCR, in wild-type (+/+), heterozygotic (*me*/+) or homozygotic *motheaten* (*me/me*) backgrounds. We crossed DO11.10 TCR transgenic mice, which express a TCR specific for chicken ovalbumin (OVA) peptide (25) onto the *motheaten* background. Analyses of

thymocytes from the progeny of these mice demonstrates that SHP-1 regulates both positive and negative selection. Data obtained from expression of a dominant negative mutant of SHP-1 in the BYDP hybridoma cell line confirm that SHP-1 helps to control TCR responsiveness in a cell-autonomous manner and suggest that one way SHP-1 negatively regulates TCR signaling might be via the regulation of Lck activity.

Methods

Cell culture and generation of cell lines

BYDP cells (26) and A20 cells (obtained from T. Braciale, University of Virginia) were grown in RPMI 1640 medium supplemented with 10% FCS, 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, and antibiotics (complete medium). Stable cell lines were generated by co-transfecting BYDP cells with the expression vector pEBG alone or pEBG constructs, into which wild-type or mutant SHP-1 had been inserted, together with the plasmid pMHneo (27), which confers G418 resistance. pEBG (28) drives expression of glutathione-S-transferase (GST)-tagged proteins under the control of the elongation factor 1-α promoter. Wild-type SHP-1 (SHP-1 wt) and a mutant of SHP-1 containing a deletion in its phosphatase domain (amino acids 451–475) [SHP-1 ΔP] (29) were subcloned from pGEX2T-SHP-1 (30) [*Bst*BI (5′) and *Avr*II (3′)] into pEBG [*Bst*BI (5′) and *Spe*I (3′)]. The parental pEBG expression vector was used as a control. Clones were selected for G418 resistance (1.5 mg/ml) and expression of exogenous SHP-1 was confirmed by immunoprecipitation followed by immunoblotting. At least five independent clones were generated for each construct, three of which were analyzed in detail and displayed similar phenotypes. Data for a representative set of clones are shown in Fig. 1.

Mice

C57BL/6 *me*/+ (originally obtained from the Jackson Laboratory, Bar Harbor, ME) and DO11.10 TCR transgenic BALB/c (generously provided by M. McDuffie, University of Virginia) mice were bred in our colony. Genotypes were determined by PCR, using the primer pairs: 5′-GGTGACTTCTCCCTCTCA-GTCAG-3′ and 5′-GTGGGGTCCGAGCAGTTCAGTG-3′ for the *me* allele, and 5′-TGGCTCTACAGTGAGTTTGGT-3′ and 5′-CAGGAGGGATCCAGTGCCAGC-3′ for the DO11.10 TCR. DO11.10 TCR Tg mice (+/+:Tg) were intercrossed with mice heterozygotic for the *me* allele (*me*/+). F_1 *me*/+ mice carrying the TCR transgene (*me*/+:Tg) were backcrossed to DO11.10 TCR Tg mice to obtain mice homozygotic for the H-2^d haplotype. F_2 *me*/+:Tg mice were then either intracrossed to obtain six possible genotypes for further analysis (+/+:non-Tg, *me*/+:non-Tg, *me/me*:non-Tg, +/+:Tg, *me*/+:Tg and *me/me*:Tg) or successively backcrossed to DO11.10 TCR Tg mice to obtain mice that were increasingly congenic. After each round of backcrossing, mice containing the desired genotype were either intracrossed or backcrossed. All genotypes were obtained in the expected Mendelian distribution. Mice aged 15–19 days were used throughout the study.

Immunoprecipitation and immunoblotting

Cells (3.5×10^6) were lysed in 0.35 ml lysis buffer (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.5 % NP-40, 1 mM Na_3VO_4, 10 mM NaF) containing leupeptin (10 μg/ml), aprotinin (1 μg/ml), pepstatin A (1 μg/ml), antipain (1 μg/ml) and PMSF (20 μg/ml). Cell lysates were clarified by centrifugation for 30 min at 100,000 *g* at 4°C. Then 3 μg of affinity-purified polyclonal rabbit anti-SHP-1 antibodies (14) was added to cleared lysates for 2 h at 4°C. Immune complexes were collected by adding 50 μl of Protein A–Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) for 30 min at 4°C, followed by five washes with lysis buffer. Samples were resolved by SDS–PAGE and electroblotted onto Immobilon P (Millipore, Bedford, MA). Immunoblots were blocked for 1 h at room temperature with 5% dry milk in TBST (10 mM Tris–HCl, pH 8.0, 150 mM NaCl and 0.05% Tween 20) before primary antibodies (anti-SHP-1, polyclonal serum) were added at 1:1000 dilution for 1 h. After three washes in TBST, secondary antibodies (1:7500 dilution; anti-rabbit-horseradish peroxidase; Amersham Pharmacia Biotech) were added for 1 h. After another five washes, immunoblots were developed using enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech).

Lck kinase assays

BYDP cells (5×10^6) were stimulated as described previously (14) with anti-CD3 (1 μg/ml) plus anti-CD4 (1 μg/ml) antibodies for the indicated times. Stimulated cells were lysed in 0.3 ml Brij 96 lysis buffer (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 10 mM sodium pyrophosphate, pH 7.6, 1% Brij 96, 1 mM Na_3VO_4 and 10 mM NaF) containing leupeptin (10 μg/ml), aprotinin (1 μg/ml), pepstatin A (1 μg/ml), antipain (1 μg/ml) and PMSF (20 μg/ml). CD4-associated Lck was immunoprecipitated from clarified lysates, prepared as described above, by adding 50 μl of Protein A–Sepharose for 1 h. The immune complexes were washed 3 times in wash buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM Na_3VO_4 and 5 mM NaF) and once in kinase reaction buffer (5 mM HEPES, pH 7.4, 5 mM *p*-nitrophenyl phosphate and 10 mM $MnCl_2$). Kinase reactions were performed in a total volume of 25 μl of kinase buffer containing 5 μCi of [γ-^{32}P]ATP for 10 min at 30°C. Reaction products were resolved by SDS–PAGE, transferred onto Immobilon P as described above and subjected to autoradiography.

IL-2 assay

Ninety-six-well plates were coated with the appropriate concentrations of anti-CD3 antibody overnight at 4°C and washed 3 times with PBS. BYDP-derived cell lines were plated in triplicate at 5×10^4/well in 200 μl complete medium and incubated for 24 h. Supernatants (125 μl) were harvested and immediately frozen at –70°C. IL-2 bioassays were performed by using IL-2-dependent CTLL cells. CTLL cells were washed 4 times in PBS, plated in 96-well plates (5×10^3/well in 50 μl complete medium), treated with 50 μl of BYDP supernatant (undiluted, 1:2, 1:4, 1:8, 1:16 and 1:32 dilutions in complete medium) for 20 h and then pulsed for 4 h with 1 μCi of [^{3}H]Thymidine. [^{3}H]Thymidine incorporation was measured as described above. All assays were performed in triplicate.

Flow cytometric analysis

Total thymocytes (2×10^6) were stained with antibodies to the indicated surface markers in PBS supplemented with 3% BSA and 0.1% sodium azide (FACS juice). All antibodies were

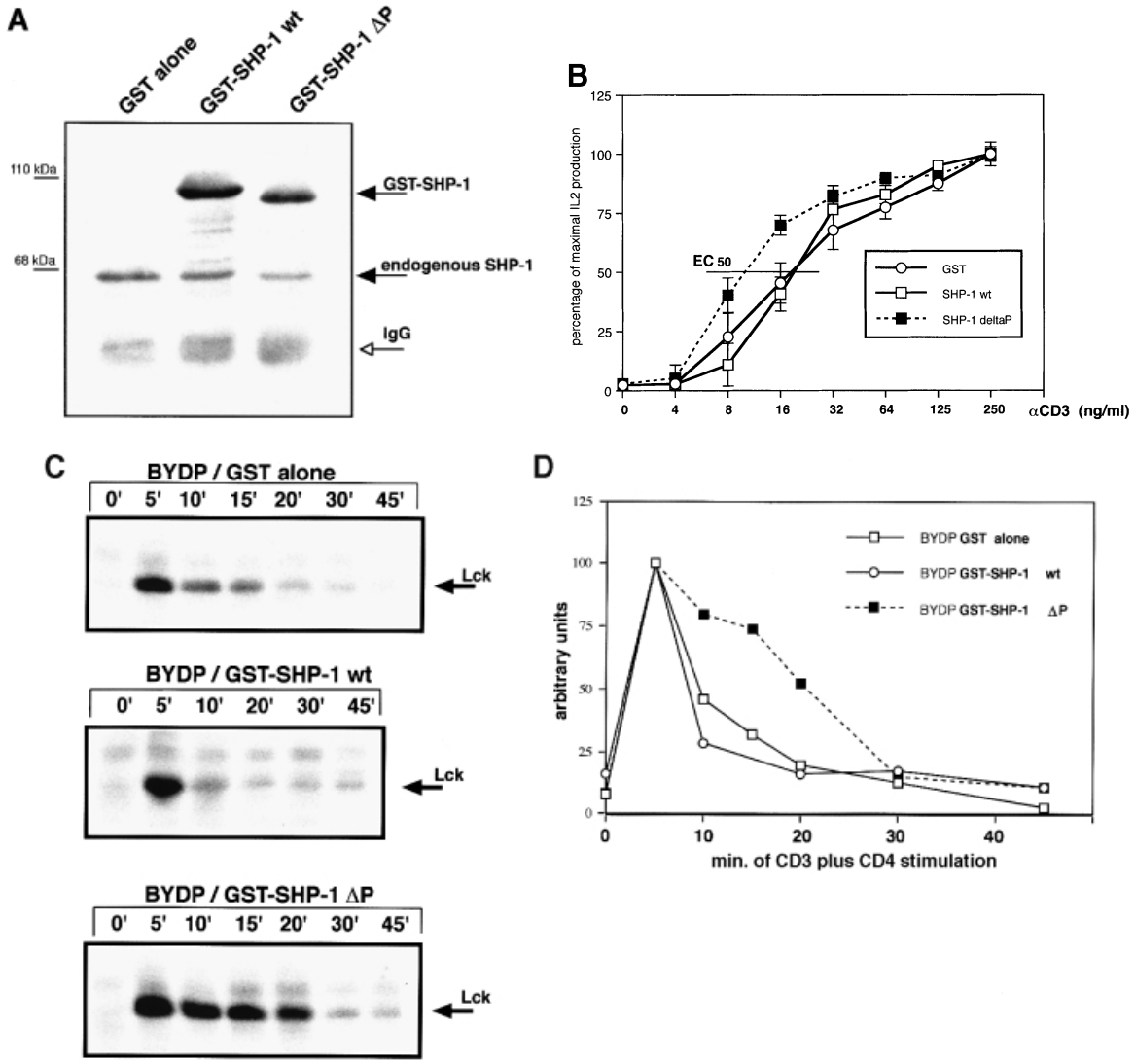

Fig. 1. Dominant negative SHP-1 confers hyper-sensitivity to TCR stimulation. (A) Generation of BYDP cell lines expressing GST, GST-SHP-1 wt or GST-SHP-1 ΔP. BYDP cells (3.5×10^6) stably transfected with the indicated constructs were lysed and immunoprecipitated with polyclonal anti-SHP-1 antibodies. Immunoprecipitates were resolved by 8% SDS–PAGE and subjected to anti-SHP-1 blotting. The positions of endogenous SHP-1 and the GST-fusion proteins are marked. (B) IL-2 bioassay. BYDP cells stably transfected with GST, GST-SHP-1 wt or GST-SHP-1 ΔP were stimulated with the indicated concentration of anti-CD3 and the amount of IL-2 produced was measured by CTLL proliferation. The data are presented as percentage of maximal IL-2 production at the highest anti-CD3 concentration. Error bars represent SEM. Where no error bars are visible, the error was smaller than the symbol for that point. (C) Lck kinase assay. BYDP cells (5×10^6) were stimulated with anti-CD3 plus anti-CD4 for the indicated times and CD4-associated Lck activity (autophosphorylation) was measured by immune complex kinase assay. (D) Quantitation of Lck activity. The intensities of the Lck autophosphorylated bands (from C) were quantitated by densitometry and their levels relative to their individual maximal phosphorylation (at 5 min) are indicated.

used at 2 ng/ml. CD4–phycoerythrin, CD8–FITC, $V_\beta8$–biotin (clone MR5-2), CD69–biotin and streptavidin–CyChrome were purchased from PharMingen (San Diego, CA); CD3–biotin and anti-mouse–FITC were purchased from Southern Biotechnology (Birmingham, AL). KJ1-26 was kindly provided by M. McDuffie, University of Virginia. To analyze stained thymocytes, 25,000 events were collected on a FACSCalibur instrument calibrated with CaliBRITE beads using CellQuest software for collection and subsequent analyses (Becton Dickinson, Mountain View, CA). Analyses were carried out on live thymocytes (>95%) as defined by forward and side angle scatter. For *ex vivo* deletion assays, 30,000 events were collected and analyses were carried out on gated live thymocytes (15–20%).

Ex vivo deletion assay and Annexin V staining

Ex vivo deletion assays were performed following a previously described protocol (31). Briefly, thymocytes (1×10^6) were co-cultured with 1×10^5 A20 B cells in the presence of the indicated concentrations of OVA peptide in complete medium. After 20 h, cells were harvested into FACS juice and analyzed by flow cytometry. For direct assessment of OVA peptide-

induced apoptosis, cells were harvested after 8 h, stained with PE-labeled Annexin V (PharMingen) and analyzed by flow cytometry. Gates for the analyses were set so that all thymocytes were included but A20 cells excluded.

Proliferation assay

Proliferation assays were performed as described previously (20). Briefly, for antibody stimulations, 96-well plates were coated with the indicated concentrations of anti-CD3 antibody (145-2c11; Southern Biotechnology, Birmingham, AL) overnight at 4°C and washed 3 times with PBS. Thymocytes were plated in triplicate at 5×10^4/well in 200 μl complete medium, supplemented with 30 U IL-2/ml and incubated for 72 h before they were pulsed with 1 μCi of [^{3}H]Thymidine for 18 h. [^{3}H]Thymidine incorporation was measured using a cell harvester (Skatron, Sterling, VA). The same procedure was used for antigen stimulation except that the indicated concentrations of OVA peptide (ISQAVHAAHAEINEAGR, synthesized at the Biomolecular Research Facility, University of Virginia; 10 ng/ml = 5.6 nM) were added directly to thymocytes on 96-well plates without pre-coating and without addition of IL-2. For proliferation assays with added antigen presenting cells (APC), 2×10^6 irradiated (2000 rad) total splenocytes (from a DO11.10 TCR transgenic BALB/c mouse) per well were added to the thymocytes, along with the indicated concentrations of ovalbumin (Sigma, St Louis, MO).

Results

SHP-1 is a cell-autonomous negative regulator of TCR signaling

We have previously shown that, when assayed for proliferation and IL-2 production, thymocytes from *me/me* mice are hyper-responsive to TCR stimulation compared to normal thymocytes (20), suggesting hyper-sensitive TCR signal transduction pathways. To directly assess whether there is a cell-autonomous hyper-sensitivity of TCR signaling in the absence of functional SHP-1, we utilized the BYDP hybridoma line, which has been shown to mimic various aspects of thymocyte signaling (26). We previously used this line to define biochemical events involving SHP-1 following TCR stimulation (14). BYDP cell lines stably expressing GST-tagged versions of either wild-type SHP-1 (SHP-1 wt) or a phosphatase-inactive, dominant negative mutant of SHP-1 (SHP-1 ΔP) were generated. The wild-type and mutant GST-SHP-1 constructs were expressed at levels ~5-fold higher than endogenous SHP-1, as assessed by immunoblotting (Fig. 1A). Data of one clone for each line are shown here as a representative of at least three independent clones. Each line was stimulated with a range of concentrations of anti-CD3 antibody and IL-2 production was measured. As shown in Fig. 1(B), an ~2-fold lower concentration of anti-CD3 was sufficient to evoke half-maximal IL-2 production by the line expressing dominant negative SHP-1 compared to the lines expressing GST-SHP-1 or GST alone.

In our previous study, we showed that when stimulated by co-cross-linking of the TCR together with CD4, thymocytes from *me/me* mice displayed prolonged activation of the *src* family kinase Lck compared to normal thymocytes (20). We tested whether this was reproduced in our model system and could therefore be attributed to a hyper-sensitivity of the TCR–CD4 co-receptor signaling pathway. BYDP cells expressing the different SHP-1 constructs were co-stimulated with anti-CD3 plus anti-CD4 and CD4-associated Lck kinase activity was assayed. All three cell lines showed a similar pattern of Lck activation within 5 min following stimulation. However, the inactivation profile was strikingly different between these lines. In GST alone- or GST-SHP-1 wt-expressing BYDP cells, Lck kinase activity was significantly diminished (by ~50%) within 10 min of stimulation. In contrast, the dominant negative GST-SHP-1 ΔP-expressing cells showed sustained Lck activity (Fig. 1C and D). This result is similar to our previous observation that thymocytes from *me/me* mice show prolonged Lck activation upon TCR–CD4 stimulation (20). Since expression of a dominant negative mutant of SHP-1 directly causes TCR hyper-sensitivity in a cell-autonomous manner, these results suggest that the primary defect in *me/me* thymocytes with respect to TCR stimulation is at the level of TCR signaling itself.

Evidence that SHP-1 plays a role in thymocyte development by influencing the thresholds for positive and negative selection

To assess whether the negatively regulatory effect of SHP-1 on TCR signaling translates into a role for SHP-1 in thymocyte development, we examined the progeny of crosses between DO11.10 TCR Tg and *me*/+ mice. DO11.10 mice express a transgenic TCR specific for a fragment of chicken ovalbumin peptide (amino acids 323–339) in the context of I-A^d. Because the antigen specific for the DO11.10 TCR is well defined, dose–response studies on thymocytes expressing the transgene can be performed to monitor TCR responsiveness either *in vivo* or *ex vivo*. We noticed that some of the early generation *me*/+:Tg and *me/me*:Tg mice showed several inconsistent and unusual phenotypes with respect to their thymocyte composition (data not shown). However, in later generations, as mice became increasingly congenic, the phenotypes of different animals bearing the same genotype were quite consistent. The observed inconsistent phenotypes in early generations were most likely due to segregating modifier loci in the C57BL/6 background (present in the *me*/+ mouse used for the initial cross). Therefore, all data presented here are derived from mice of F_5 or later generations (four rounds of backcrossing; theoretically, ⩽3% genetic contribution by C57BL/6).

Effects in homozygotic me/me *TCR Tg mice*

Thymocytes from the various mice derived from intracrosses were analyzed for their surface marker expression (Fig. 2 and Table 1). As expected from previous studies (20,21,23,24), thymuses from +/+:DO11.10 TCR Tg (+/+:Tg) mice showed a normal composition with respect to the percentages of DN ($CD4^-CD8^-$), DP ($CD4^+CD8^+$) and SP ($CD4^+CD8^-$ and $CD4^-CD8^+$) subpopulations (Fig. 2A). A similar (normal) composition was observed for non-Tg thymi, independent of their genotype at the SHP-1 locus (Fig. 2D and bottom half of Table 1). These data are consistent with our previous observation that *me/me* mice have overall normal thymocyte subpopulations (20). However, in our analyses of a large number of mice, we observed that in the presence of the DO11.10 transgenic TCR, thymocytes from *me/me* mice

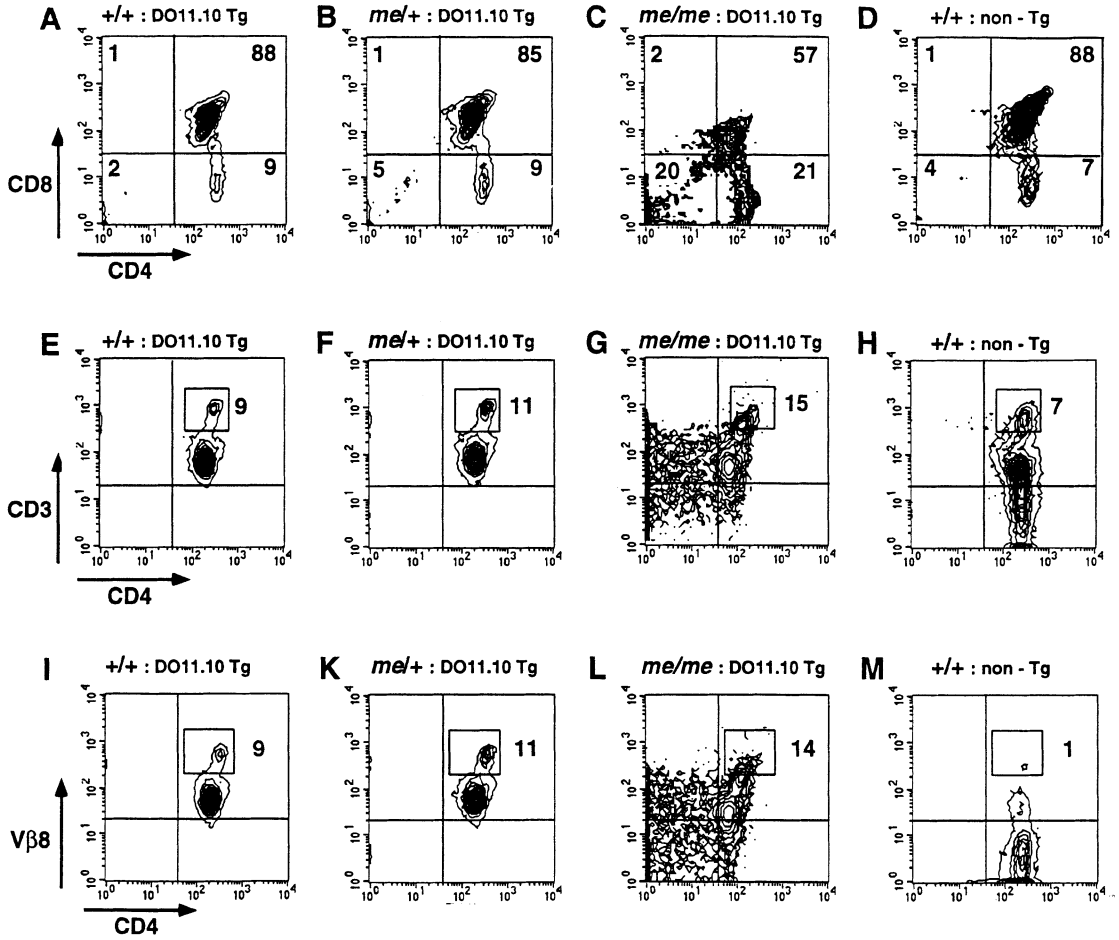

Fig. 2. Flow cytometric profiles. Representative flow cytometric analyses of thymocytes from littermates (fifth backcross generation) carrying the indicated genotypes. Thymocytes were triple-stained with CD4–PE, CD8–FITC and CD3–(biotin–streptavidin)–CyChrome (A–H) or with CD4–PE, CD8–FITC and $V_\beta 8$–(biotin–streptavidin)–CyChrome (I–M). (A–D) CD4 versus CD8 profiles. Profiles from non-Tg *me*/+ or *me/me* thymocytes are very similar to the presented profile (D) of non-Tg +/+ thymocytes (data not shown, but see Table 1). Numbers represent the percentage of cells in the different regions. (E–H) CD4 versus CD3 profiles. Note that the $CD4^+CD3^{low}$ population in +/+:non-Tg mice, which is absent in the Tg mice, reflects $CD4^+CD8^+TCR^{low–med}$ thymocytes, a normal population in thymocyte development, which is missing in TCR Tg mice due to their pre-arranged TCR. (I–M) CD4 versus $V_\beta 8$ profiles. The boxed regions of $CD3^{high}$ or $V_\beta 8^{high}$ thymocytes correspond to the $CD4^+$ SP population. Numbers represent the percentages of cells in each indicated region.

revealed abnormalities, manifested as the following unusual phenotypes.

Decreased number of DP cells. Compared to +/+:Tg mice (n = 32), *me/me*:Tg mice (n = 38) showed a dramatic decrease in the number of total thymocytes [1.5×10^8 (+/+) versus 0.36×10^8 (*me/me*), ~24%] (Table 1). Flow cytometric analyses revealed that this reduced cellularity was almost entirely due to a dramatic loss of DP cells (Table 1, and Fig. 2A and C). Moreover, the remaining DP population in the *me/me*:Tg mice displayed decreased levels of CD4 and CD8 surface expression. The dramatic decrease in DP thymocytes, concomitant with the relatively constant cell numbers of the other thymic subpopulations and additional *ex vivo* deletion studies (see below, Fig. 4), support the conclusion that the *me/me*:Tg mice display increased negative selection even without exposure to specific antigen. This is consistent with our initial hypothesis that in the absence of SHP-1, an antigen recognized with moderate avidity, which 'normally' would lead to positive selection, instead is now perceived as a high-avidity antigen causing deletion. These data indicate that SHP-1 helps to set the threshold for negative selection.

Relative increase of $CD4^+CD8^-$ SP cells. Consistently, we observed that in *me/me*:Tg mice, there was a 2- to 4-fold increase in the percentages of $CD4^+$ SP cells (Table 1, and Fig. 2A and C), as compared to +/+:Tg mice. Selection of $CD4^+$ SP cells is consistent with the MHC class II specificity of the DO11.10 TCR, suggesting specific positive selection of these thymocytes in *me/me*:Tg mice. The observed *relative* increase could be fully accounted for by the decreased total cellularity due to the dramatic loss of DP cells. Although the absolute number of $CD4^+$ SP cells was ~70% in a *me/me*:Tg mouse compared to a +/+:Tg mouse, after normalizing for the decreased body mass of the *motheaten* mouse (about two-thirds that of its normal littermate), the number of $CD4^+$ SP cells was actually constant between a +/+:Tg and a *me/me*:Tg mouse. This was interesting given that *me/me*:Tg mice

Table 1. Summary of flow cytometric data

Genotype	Thymocyte subpopulation	+/+		*me*/+			*me*/*me*		
		%	*N*	%	*N*	% relative to +/+	%	*N*	% relative to +/+
DO11.10 Tg	no. of analyzed animals	*n* = 32		*n* = 85			*n* = 38		
	$CD4^+CD8^+$	80 ± 2	12.0×10^7	71 ± 2	9.2×10^7	77	49 ± 3	1.8×10^7	15
	$CD4^-CD8^-$	9 ± 1	1.4×10^7	14 ± 1	1.8×10^7	129	28 ± 3[a]	1×10^{7a}	
	$CD4^+CD8^-$	7 ± 1	1.0×10^7	12 ± 1	1.6×10^7	160	19 ± 2	0.7×10^7	70
	$CD4^-CD8^+$	4 ± 1	0.6×10^7	3 ± 1	0.4×10^7	67	4 ± 1	0.1×10^7	17
	total thymic cell no.	$1.5 \pm 0.1\times10^8$		$1.3 \pm 0.1\times10^8$		87	$0.36 \pm 0.1\times10^8$		24
Non-Tg	no. analyzed animals	*n* = 6		*n* = 12			*n* = 11		
	$CD4^+CD8^+$	85 ± 1	14.5×10^7	85 ± 1	12.8×10^7	88	80 ± 2	8.0×10^7	55
	$CD4^-CD8^-$	4 ± 1	0.6×10^7	4 ± 1	0.6×10^7	100	6 ± 1	0.6×10^7	100
	$CD4^+CD8^-$	10 ± 1	1.7×10^7	10 ± 1	1.5×10^7	88	13 ± 1	1.3×10^7	76
	$CD4^-CD8^+$	1 ± 1	0.2×10^7	1 ± 1	0.1×10^7		1 ± 1	0.1×10^7	
	total thymic cell no.	$1.7 \pm 0.1\times10^8$		$1.5 \pm 0.1\times10^8$		88	$1.0 \pm 0.1\times10^8$		59

Thymocytes from mice carrying the indicated genotypes were analyzed for CD4 and CD8 surface expression. The relative (%) and absolute numbers (*N*) of thymocytes displaying the indicated phenotype are shown. Errors represent the SEM. For easier comparison, the cell numbers of *me*/+ and *me*/*me* mice relative to the cell numbers of +/+ mice are shown in the last column of each panel.

[a]The majority of the $CD4^-CD8^-$ population in the *me*/*me*:Tg mice were $CD3^+$ and $V_\beta8^+$ (Fig. 3G and L) indicating that this population is different from the DN populations in mice of other genotypes.

exhibit a big loss of DP cells, some of which would 'normally' give rise to $CD4^+$ SP cells. It was therefore conceivable that we would also observe a loss of $CD4^+$ SP cells. Indeed, this was the case for the small population of non-transgenic TCR expressing $CD8^+$ SP cells.

To further characterize the thymocyte subpopulations, thymocytes were triple-stained for CD4, CD8 and CD3 (CD4 versus CD3; Fig. 2E–H). The majority of the $CD4^+$ SP thymocytes in the +/+:Tg as well as the *me*/*me*:Tg mice were $CD3^{high}$ (and $CD69^{high}$, data not shown), indicating that these are indeed mature, positively selected thymocytes. Analyses of TCR β expression on these $CD4^+$ SP cells showed that they express the transgenic $V_\beta8$ chain (CD4 versus $V_\beta8$; Fig. 2I–M) (see below for comparison with staining by KJ1-26, a clonotypic anti-DO11.10 TCR antibody which recognizes the transgene-specific α and β chains of the TCR).

Our results suggest that, in addition to helping to set the threshold for negative selection, SHP-1 also plays a role in setting the threshold which determines whether a cell is 'neglected' or positively selected. In the absence of SHP-1, both thresholds are lowered leading to enhanced negative selection and 'leftward-shifted' positive selection.

TCR^+ DN cells. In addition to the relative increase in $CD4^+$ SP cells, thymocytes from *me*/*me*:Tg mice also showed an ~3- to 5-fold increase in the percentage of DN cells, which lack both CD4 and CD8 expression (Fig. 2 C). Interestingly, we found that, unlike in normal mice, the majority of these DN thymocytes in *me*/*me*:Tg mice were $CD3^+$ and $V_\beta8^+$ (Fig. 2G and L). These data indicated that these cells were not 'typical' DN cells or NK cells arising in the *me*/*me* background. Since TCR expression on these cells was medium to high and they were also $CD69^{high}$ (data not shown), it is likely that these TCR^+ DN thymocytes were positively selected or at least had been stimulated via the TCR, either in the absence of co-receptors or in the presence of CD4 and CD8 whose surface expression was then lost during further maturation. At the time point that thymic compositions were analyzed (15–19 days post-natal), the majority of the DN cells in *me*/*me*:Tg expressed the TCR and was therefore part of this unusual subpopulation, only a relatively small fraction of thymocytes were 'true' DN cells.

To analyze the thymocytes not only for expression of the transgenic $V_\beta8$ chain, but also for expression of the complete transgenic TCR (α and β chain), cells were stained with KJ1-26, a clonotypic anti-DO11.10 TCR antibody (Fig. 3). For +/+:Tg thymocytes, staining with KJ1-26 overlapped the $V_\beta8$ staining (Fig. 3A), indicating that all of the cells express both the α and β chain of the transgenic TCR. With respect to $V_\beta8$ staining *per se*, *me*/*me*:Tg thymocytes showed an increase in $V_\beta8^{high}$ and a decrease in $V_\beta8^{med}$ staining (Fig. 3B and C). This is consistent with the increase in $CD4^+$ SP and the decrease in DP cells caused by the enhanced negative and 'leftward-shifted' positive selection in *me*/*me*:Tg mice. In general, *me*/*me*:Tg mice also displayed similar staining for $V_\beta8$ and KJ1-26. We observed some variation in different mice with the *me*/*me*:Tg genotype, most likely due to slight genetic variations. About 50% of the *me*/*me*:Tg mice showed an almost identical profile for $V_\beta8$ and KJ1-26 staining (Fig. 3B), whereas the other half displayed a small decrease of KJ1-26^{med} staining compared to $V_\beta8^{med}$ (Fig. 3C), indicating that a small portion of thymocytes from these mice expressed medium levels of a TCR composed of the transgenic β chain paired with endogenous α chains.

Effects seen in heterozygotic me/+ TCR Tg mice

Interestingly, heterozygotic *me*/+:Tg mice displayed a phenotype intermediate between +/+:Tg and *me*/*me*:Tg mice with an absolute increase in $CD4^+$ SP cells (>50%) and a 25% loss of DP cells (Table 1). This suggested that even a 50% reduction of SHP-1 caused a change in the threshold for

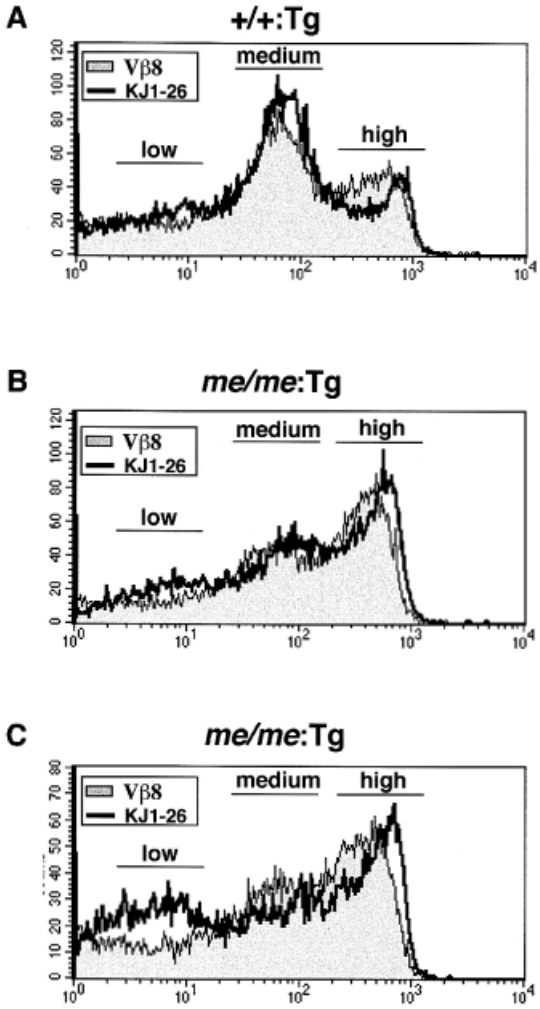

Fig. 3. $V_\beta 8$ and KJ1-26 profiles. Representative flow cytometric analyses of thymocytes from littermates carrying the indicated genotypes. Panels (B) and (C) show the profiles of two different mice representing the observed variation within the *me/me*:Tg population. Thymocytes were either stained for $V_\beta 8$ (shaded area) or for α:β transgenic TCR (KJ1-26) (black line) surface expression. Areas of TCR low, medium or high expression are marked.

thymocyte selection. In the case of the *me*/+ mice, the absolute increase in $CD4^+$ SP cells was most likely due to the only slightly reduced number of DP cells serving as precursors. In contrast in the *me/me* mice, the number of DP thymocytes was so strongly reduced that even a 'leftward-shifted' positive selection generated only a limited number of $CD4^+$ SP cells.

Thymocytes from me/me mice execute negative selection at lowered antigen concentrations

The above data on DO11.10 TCR Tg mice suggested that there was a change in positive and negative thymocyte selection thresholds in *me/me* progeny compared with +/+ mice, which was observable even in the absence of the TCR-specific OVA peptide. To further test the hypothesis that SHP-1 helps control positive/negative selection by regulating the strength of TCR-mediated signals, we analyzed thymocyte responsiveness to various dosages of OVA peptide. We chose not to administer peptide *in vivo* by injection since the increased numbers of macrophages in the *me/me* mouse would add uncontrollable variables to the experiment, such as clearance, delivery and presentation of the peptide. Instead, we performed *ex vivo* assays using primary thymocytes from mice of the various genotypes.

Previously, it has been shown that exposure of immature thymocytes to their specific antigen *ex vivo* leads within a day to deletion due to negative selection (31). We hypothesized that if SHP-1 plays a regulatory role in this process, the critical concentration of antigen needed for negative selection should be lowered in the absence of SHP-1. Thymocytes from +/+:Tg and *me/me*:Tg mice were incubated with increasing concentrations of OVA peptide in the presence of A20 cells as APC and thymic composition was examined the following day. In the absence of A20 cells, no deletion was observed at concentration $\leqslant$100 nM. As described previously (31) in the presence of A20 cells, there was 'spontaneous deletion' even in the absence of antigen; ~40–50% in the *me/me*:Tg and 30–40% in +/+:Tg thymocyte populations. Since this slightly increased deletion of *me/me*:Tg thymocytes is dependent on the presence of the antigen presenting A20 cell, it might have been caused by stimulation with a peptide derived from the A20 cells. In the hyper-sensitive *me/me* background, this stimulation could have been strong enough to cause enhanced negative selection. As shown in Fig. 4(A and B), thymocytes from +/+:Tg mice (top row of each panel in Fig. 4A) do not show deletion of the immature $CD4^+CD8^+$ DP population <7.5–10 nM OVA peptide, consistent with the previous report (31). In contrast, *me/me*:Tg thymocytes (bottom row of each panel in Fig. 4A) displayed significant OVA-specific deletion even in the 1–7.5 nM range. In this assay, negative selection can be observed not only as a decrease in the DP subpopulation, but also as a shift within the DP profile from $CD4^{high}CD8^{high}$ towards $CD4^{low}CD8^{low}$ as a transitional stage towards death (31). We then confirmed that the observed loss of DP thymocytes reflected actual negative selection of these thymocytes, rather than a down-modulation of the co-receptors CD4 and CD8 caused by stimulation of the TCR (33). Thymocytes from +/+:Tg and *me/me*:Tg mice were incubated with increasing concentrations of OVA peptide presented by A20 cells and induced cell death was measured directly by Annexin V staining. Again, *me/me*:Tg thymocytes were hyper-responsive and exhibited cell death due to negative selection at much lower concentration of OVA peptide than +/+:Tg thymocytes (Fig. 4C). These results, showing increased negative selection in the *me/me* background confirm that SHP-1 acts in a cell-autonomous manner to set the threshold for negative selection and that in the absence of SHP-1, a decreased dose of antigen is needed to induce cell death, i.e. a 'leftward-shift' in the dose response is observed. These *ex vivo* deletion data are fully consistent with the increased negative selection observed *in vivo*.

Hyper-sensitive proliferative response of thymocytes from me/me *TCR Tg to specific antigen*

The differences in thymocyte composition between +/+:Tg and *me/me*:Tg mice suggested that negative and maybe positive selection are affected by the absence of SHP-1

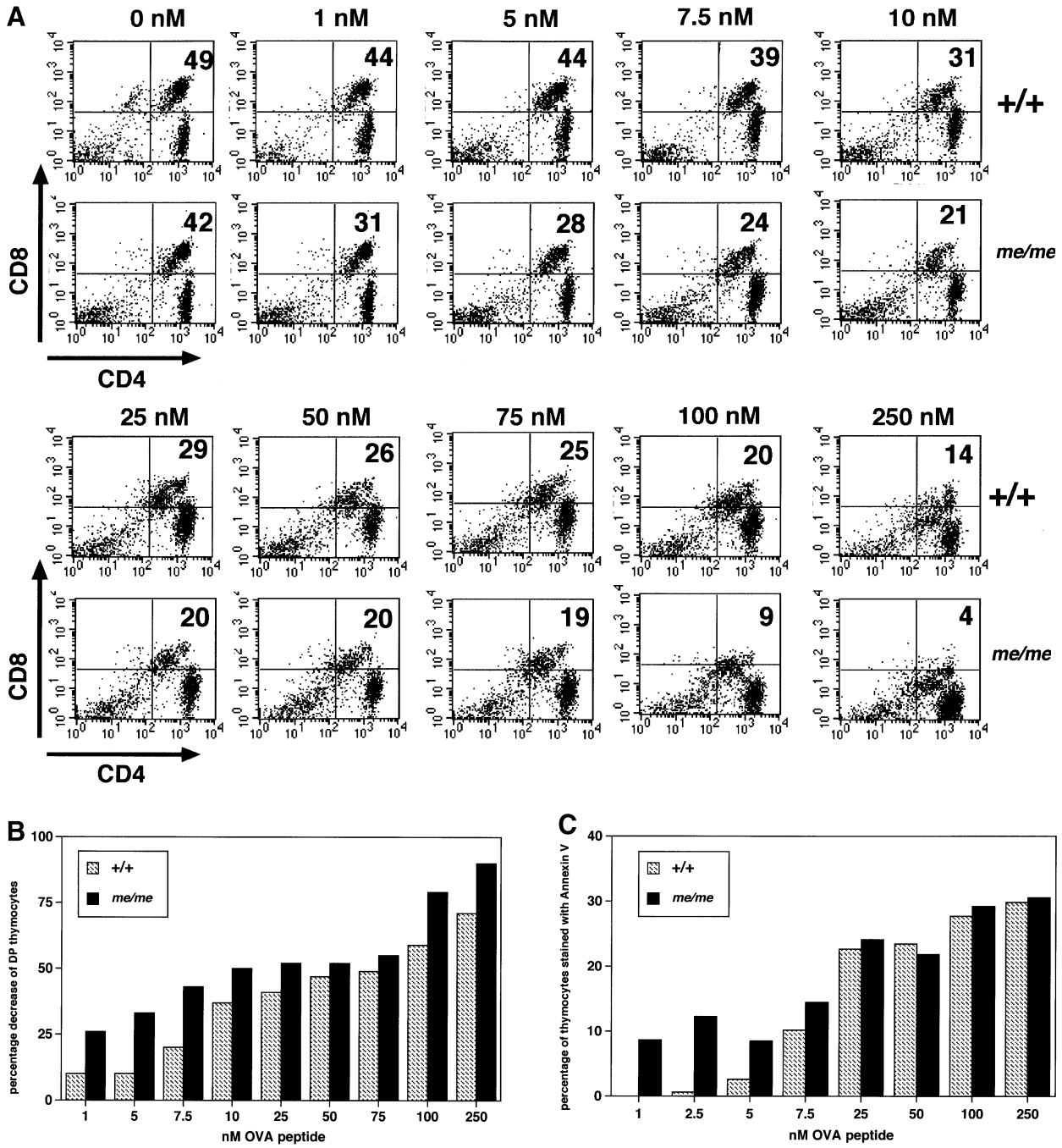

Fig. 4. Thymocytes from *me/me* mice exhibit lowered threshold for negative selection. (A) Flow cytometric analyses of thymocytes from +/+:Tg (top row of each panel) and *me/me*:Tg (bottom row of each panel) mice incubated with the indicated concentrations of OVA peptide in the presence of A20 B cells as APC. Harvested thymocytes were stained with CD4–PE and CD8–FITC. Numbers represent percentages of $CD4^+CD8^+$ DP thymocytes. At concentrations of ⩾100 nM OVA peptide, some (10–20%) deletion occurred in the *me/me* without addition of A20 cells. (B) Graphical representation of the percentages of $CD4^+CD8^+$ DP thymocytes deleted (derived from data in A) relative to the population, which was not subjected to OVA peptide-induced deletion (0 nM OVA peptide; 49 for +/+ and 42 for *me/me*). (C) Graphical representation of percentages of thymocytes which showed OVA peptide-induced staining with Annexin V. Numbers show percentages above background staining (incubation without OVA peptide: 20% for +/+:Tg and 18% for *me/me*:Tg). (B and C) The data shown are representative of at least three independent experiments. No error bars are shown because the absolute numbers are varying between experiments, dependent on the initial percentages of DP thymocytes. However, the dose–response curves are similar.

as well as a possible hyper-proliferation of the $CD4^+$ SP subpopulation. To test the responsiveness of mature, positively selected thymocytes, we measured their proliferative response to anti-CD3 stimulation. Similar to our previous observations using thymocytes from non-Tg mice (20), we observed hyper-sensitivity of the *me/me* thymocytes com-

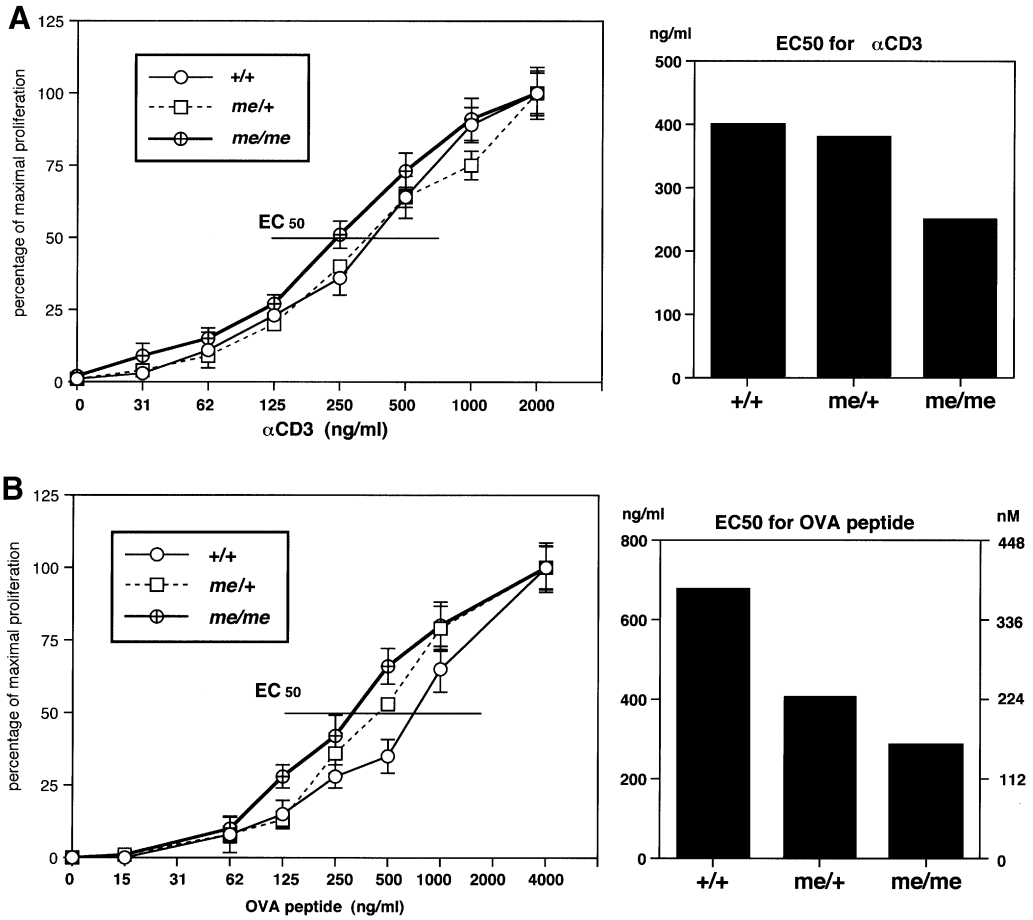

Fig. 5. Thymocytes from *me/me* DO11.10 mice are hyper-sensitive to TCR stimulation. Proliferative response of +/+, *me*/+ and *me/me* thymocytes from DO11.10 transgenic mice to increasing amounts of (A) anti-CD3 antibody (145-2c11) in the presence of IL-2 (30 U/ml) or (B) OVA peptide. The data shown are representative of experiments repeated 8–10 times, each of them consisting of five to 10 mice. Error bars represent SEM. Where no error bars are visible, the error was smaller than the symbol for that point. To facilitate the evaluation of these experiments, the EC_{50}s of the presented dose–response curves are shown as bar graphs. For comparisons with previous experiments, concentrations of used OVA peptide are indicated as ng/ml and as nM.

pared to the +/+ or *me*/+ thymocytes, which was manifested by an ~1.5- to 2-fold lower EC_{50} (~250 versus 400 ng/ml) (Fig. 5A). The data are presented as percentage of maximal stimulation, since the absolute values also depend on the number of responding cells. Based on the flow cytometric profiles (see above), this number is expected to differ between mice of the various genotypes. In general, thymocytes from *me/me* mice incorporated 1.5–2.5 times more radioactivity than +/+ or *me*/+ thymocytes. We then asked how these thymocytes respond to their specific antigen, the OVA peptide. Total thymocytes were incubated with increasing amounts of OVA peptide and proliferation was measured (Fig. 5B). As in the case of anti-CD3 stimulation, *me/me*:Tg thymocytes were hyper-sensitive compared with +/+:Tg thymocytes, with an ~2- to 2.5-fold lower EC_{50} observed (~285 versus 675 ng/ml). Interestingly, in contrast to anti-CD3 stimulation where +/+ and *me*/+ thymocytes were indistinguishable, thymocytes from *me*/+ mice showed an intermediate responsiveness to OVA peptide with an EC_{50} between *me/me* and +/+. This dosage-dependent epistatic interaction between the SHP1 and TCR loci provides additional genetic evidence that the two proteins act in the same pathway.

Since the above proliferation assays had been performed with total thymocytes, intrinsic dendritic cells and macrophages had functioned as APC for the OVA peptide. Therefore, the possibility remained that the observed hyper-sensitivity of *me/me* thymocytes to OVA peptide was due to differences in antigen presentation, rather than actual hyper-sensitivity of the TCR in the *me/me* background. To address this possibility, we measured proliferation of thymocytes in response to specific antigen presented by the same, defined wild-type APC. Total splenocytes from +/+:Tg mice were irradiated and added as APC to the transgenic thymocytes, along with increasing amounts of whole ovalbumin rather than OVA peptide. Whole ovalbumin was chosen as the antigen since thymocyte preparations were not able to respond to it, most likely due to their limited numbers of intrinsic APC (J. D. Carter and U. Lorenz, unpublished observation). Thus, proliferative responses seen in this experiment were wholly dependent on added APC

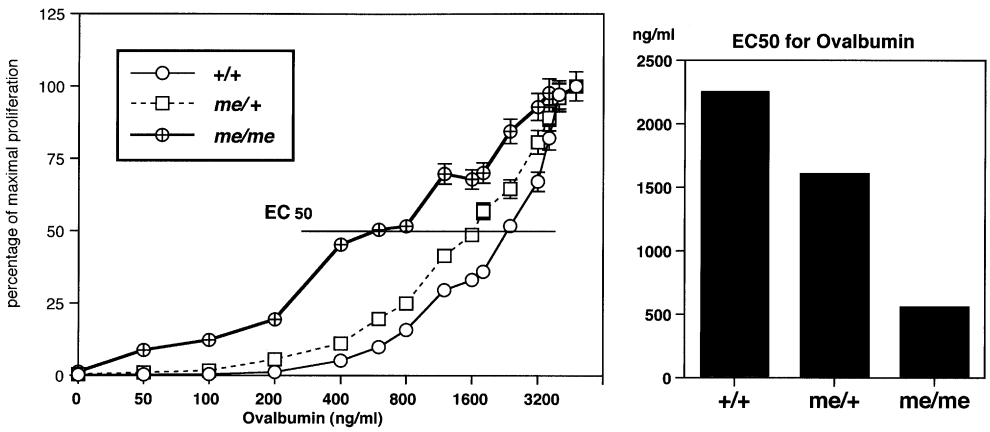

Fig. 6. Intrinsic hyper-sensitivity of TCR expressed on thymocytes from *me/me* DO11.10 mice. Proliferative response of +/+, *me*/+ and *me/me* thymocytes from DO11.10 transgenic mice to increasing amounts of whole ovalbumin, presented by irradiated splenocytes from normal mice. Without addition of splenocytes, no thymocyte proliferation in response to ovalbumin could be detected. The data shown are representative of experiments repeated 7 times, each of them consisting of five to 10 mice. Error bars represent SEM. Where no error bars are visible, the error was smaller than the symbol for that point. To facilitate the evaluation of these experiments, the EC_{50}s of the presented dose–response curves are shown as bar graphs.

derived from mice expressing wild-type SHP-1. Hyper-sensitivity of *me/me* thymocytes compared to +/+ or *me/+* thymocytes (EC_{50}s of 550 versus 1600 and 2250 ng/ml) was again observed under these conditions (Fig. 6), indicating that the antigenic stimulation of the TCR leads to an increased signal in the absence of the negative regulator SHP-1. It is interesting that the hyper-sensitivity of the *me/me* thymocytes was more pronounced in the presence of defined wild-type APC than in the presence of the intrinsic dendritic cells and macrophages (*cf.* Figs 5B and 6). This may indicate a less effective antigen presentation by *me/me* APC or a lower number of potential APC in *me/me* mice.

Discussion

Based on recent studies comparing the responsiveness of thymocytes from normal and *me/me* thymocytes to TCR stimulation (20,21,34), SHP-1 has been suggested to be a negative regulator of TCR signaling. To address whether the observed hyper-sensitivity to TCR stimulation was cell-autonomous and directly due to the lack of SHP-1 activity, we generated T cell lines which stably express wild-type or a dominant negative mutant of SHP-1. Functional studies of these lines showed that the observed regulatory role of SHP-1 is indeed cell-autonomous and can be directly attributed to a decrease or lack of SHP-1 activity. Remarkably when assayed for IL-2 production, the level of hyper-sensitivity produced upon expression of dominant negative SHP-1 in BYDP cells (~2-fold) is comparable to the thymocyte hyper-sensitivity observed comparing proliferation of *me/me* and normal thymocytes (20). Furthermore, our data indicate that the regulation of Lck activity, specifically of CD4-associated Lck, either directly or indirectly, is at least one mechanism by which SHP-1 acts as a negative regulator of TCR signaling thresholds.

These data and reports that thymocyte selection is influenced by the signal strength mediated via the TCR led us to hypothesize that SHP-1 might play a role in this process by affecting the signaling thresholds for positive and negative selection. To test our hypothesis *in vivo*, we used a genetic model and crossed the *motheaten* mutation into the DO11.10 transgenic TCR background. Flow cytometric analyses of progeny from these crosses revealed a number of alterations in the thymocyte phenotypes in *me/me*:Tg mice compared to +/+:Tg mice. First, there was a greatly reduced cellularity which is at least to a large part due to a dramatic loss of DP thymocytes in the *me/me*:Tg mice. A decreased number of DP cells can have five possible reasons. (i) Thymocytes arrest at the DN stage before they mature to DP cells. However, in this case, we would expect an increase in the absolute number of DN cells, which we did not observe. In contrast, the DN population was diminished (see below). (ii) Thymocytes do not expand at the DP stage. A failure to proliferate would be followed by a similar decrease in the number of SP cells. However, this was not detected in the *me/me*:Tg mice; if at all, *me/me* thymocytes seemed to hyper-proliferate (see below). (iii) A decrease in thymic precursor cells causing an overall diminished thymic cellularity. However, since this would affect all thymocytes the ratios between the different subpopulations (DN, DP, $CD4^+$ SP and $CD8^+$ SP) would not be changed. (iv) Failure to express CD4 and CD8 during the transition from DN to DP stage. It is possible that in the absence of SHP-1, a thymocyte at the transition from DN to DP (TCR^+ $CD4^-CD8^{low}$) perceives a signal mediated via the TCR as strong enough to down-regulate surface expression of CD4 and CD8, thereby generating TCR^+ DN cells as described for TCR cross-linking studies of $TCR^+CD4^-CD8^{low}$ cells (32). Interestingly, a TCR^+ DN population was observed in the *me/me*:Tg mice (see below). Since these cells never enter the DP stage, the DP population would be decreased at the same time. However, the limited number of TCR^+ DN cells (10^7) cannot by itself be the only reason for the dramatic loss in the DP population [12×10^7 (+/+) versus 1.8×10^7 (*me/*

me)]. (v) Increased cell death of DP thymocytes due to negative selection.

The DP population remaining in the *me/me*:Tg mice showed a shift from $CD4^{high}CD8^{high}$ towards $CD4^{low}CD8^{low.}$ There are two possible causes for the appearance of such a population: (i) these thymocytes down-regulated their surface expression of CD4 and CD8 in response to a TCR–CD4 stimulation as it was observed in the *ex vivo* deletion assays (Fig. 4A) or (ii) because of their low surface expression of CD4 and CD8, these cells escaped the increased ongoing negative selection and are therefore enriched in the *me/me*:Tg mice. In contrast to the decreased number of DP cells, the absolute number of $CD4^+$ SP thymocytes stayed relatively constant. Moreover, an unusual TCR^+ DN population was observed. These data, indicating increased negative selection in *me/me*:DO11.10 Tg mice, together with studies showing that thymocytes from these mice are hyper-sensitive to their specific antigen when examined in *ex vivo* deletion assays support our hypothesis that SHP-1 helps to set the threshold for negative selection and that, in its absence, the threshold is lowered.

The lack of effect on the number of $CD4^+$ SP thymocytes despite the dramatic loss of DP thymocytes can have two possible, not mutually exclusive, reasons. (i) A subpopulation of thymocytes which would have been neglected under normal conditions (in a +/+:Tg) was positively selected. Since these mature $CD4^+$ SP cells were observed without exposure to the TCR-specific OVA peptide, positive selection in *me/me*:Tg mice must have, at least partially, occurred following stimulation by endogenous peptide(s) of low avidity for the transgenic TCR. Under normal conditions, such stimulation would have led to neglect due to insufficient stimulation; however, in the absence of SHP-1, a lowered threshold for positive selection would enable antigens with 'normally' too low affinity to positively select thymocytes expressing the Tg TCR. (ii) The $CD4^+$ SP thymocytes hyper-proliferated following positive selection leading to a selective expansion of this subpopulation. Based on our *ex vivo* proliferation assays which showed a hyper-sensitivity of *me/me*:Tg thymocytes to antigen-specific stimulation, it is conceivable that at least part of the decreased DP population was compensated at the $CD4^+$ SP stage by hyper-proliferation. However, cell cycle studies showed that the percentages of $CD4^+$ SP thymocytes in G0/G1, S and G2/M phase were similar between +/+, *me/* + and *me/me* DO11.10 TCR Tg mice indicating comparable *in vivo* proliferation (J.D.Carter and U.Lorenz, unpublished observation). Therefore, it is very likely that the threshold for positive selection also was lowered in the absence of SHP-1, especially in the light of two very recent reports showing increased positive selection, one of which examined *me/me* mice expressing a MHC class I-restricted transgenic TCR (35) and one of which used mice expressing a dominant negative mutant of SHP-1 in the context of a MHC class II-restricted transgenic TCR (36).

In addition to the phenotypes described above, thymuses from *me/me*:Tg mice contained an unusual population of TCR^+ DN thymocytes. There are several possible explanations for the appearance of these cells. These cells might have been derived from a population ($TCR^+CD4^-CD8^{low}$) in transition from DN to DP which, due to the absence of SHP-1, received a strong enough signal mediated via the TCR to down-regulate surface expression of CD4 and CD8. Such a mechanism has been observed in *ex vivo* studies (32). Another possibility is that these cells were positively selected but then down-regulated CD4 expression. Alternatively, in the absence of SHP-1, the TCR alone could send a signal of sufficient strength to positively select cells which express neither CD4 nor CD8. Under normal conditions, TCR^+ DN would not receive a signal of sufficient strength and be neglected. It is also possible, that TCR^+ DN thymocytes represent a distinct lineage in Tg mice that accumulates under these specific conditions, as seen previously in male HY-TCR Tg mice (37). Further studies will be necessary to distinguish between these possibilities.

It is striking that the dramatic decrease of DP thymocytes due to negative selection observed here was not detectable in a non-Tg *me/me* mouse. There are two possible, not mutually exclusive, reasons for this observation. (i) The number of potentially different TCR with their various avidities in a non-TCR Tg mouse might not allow us to uncover such an effect because the deleted thymocytes would be continuously replaced. In contrast, in TCR Tg mice in which a major fraction of the thymocytes carry a TCR of the same avidity, an increase in deletion would be more easily detected. (ii) One of the differences between a Tg TCR and an endogenous TCR is the early expression of high levels of the Tg TCR as evidenced by the absence of a TCR^{low} population (*cf.* Figs 2E–G and 2H). It is conceivable that negative selection in a TCR Tg mouse occurs at an earlier developmental state within the DP population compared to a non-TCR Tg mouse, causing a greater apparent loss of DP thymocytes. It is important to stress that our data indicate that the 'normal' thymocyte composition in *me/me* mice only reflects the 'steady-state' level of the overall thymocyte population, but does not imply that TCR signaling with respect to thymocyte selection is normal in *me/me* mice. Instead our data predict the average affinity of a TCR for its antigen in *me/me* mice is lower than in normal mice. These differences are revealed only in the sensitized background of TCR Tg mice.

In previous studies, we and others failed to observe any abnormal/intermediate phenotype in heterozygotic *me*/+ mice, indicating that one wild-type allele of SHP-1 is sufficient to produce a normal phenotype. However in this study, we observed an intermediate phenotype for *me*/+ mice compared to +/+ and *me/me* mice when analyzed for thymic composition (Table 1 and Fig. 2), as well as when assayed for proliferative response to its specific antigen (Figs 5 and 6). This epistatic interaction between the SHP-1 and the TCR loci provides further genetic evidence that SHP-1 and the TCR are part of the same signaling pathway. Detection of this phenotype is most likely due to the sensitized background provided by the use of a TCR transgene, which allows us to reveal subtle differences in signal strengths. Similar dosage effects of signaling molecules on thymocyte selection have been elegantly demonstrated by Perlmutter and colleagues using different copy numbers of an Lck transgene (38). This is particularly interesting, since our previous studies (20) and data present here indicate that Lck might be at least one of the targets by which SHP-1 exerts its regulatory function in TCR signaling.

In addition, the fact that we observed an intermediate

phenotype for OVA peptide stimulation, but not for anti-CD3 cross-linking, emphasizes the differences between non-physiological antibody cross-linking and antigen-specific stimulation by APC. CD3 cross-linking biases towards signals delivered by TCR stimulation, whereas under physiological conditions, signals are delivered by both the TCR and the CD4–MHC complex. The observed difference is of particular interest since our data implicate the CD4-associated Lck as a target for SHP-1. A similar distinction was noted previously in a $CD45^-$ T cell line which was reconstituted with different isoforms of CD45. Certain functional differences between these isoforms were revealed upon antigen-specific stimulation but not upon anti-TCR cross-linking (39).

Interestingly, in two recent reports which addressed the role of SHP-1 in thymocytes development, SHP-1 seemed to regulate only positive selection but no change in negative selection was observed. Most likely, these differences are due to the genetic background of the mice that were analyzed and the different transgenic TCR that were used. For example, Johnson *et al*. studied the progeny of C57BL/6J mice crossed to C57BL/10 mice in the context of a MHC class I-restricted TCR (35), whereas Plas *et al*. analyzed progeny of cross between C57BL/6 and B6.AKR mice using a MHC class II-restricted TCR (36). In our studies, we used a MHC class II-restricted TCR in a >97 % congenic BALB/c background generated by at least five rounds of backcrosses. Since the endogenous antigens which 'normally' cause positive selection of these transgenic TCR are unknown, it is impossible to identify the exact differences between the mice used in the three studies. However, it is conceivable that there are differences in affinities which, in turn, affect the sensitivity of the individual mouse to the loss of a negative regulator such as SHP-1. Interestingly in a recent study, it was reported that *motheaten*^{viable} (*me*^{v}/*me*^{v}) mice, which carry a different mutated allele of SHP-1 encoding a protein with decreased phosphatase activity, show increased deletion in response to an endogenous superantigen (40). Together with our studies, this provides further evidence that SHP-1 is involved in setting the threshold for negative selection. As discussed above, we observed that the *me*/+ mouse produces an intermediate phenotype between +/+ and *me/me* mice with a more detectable effect on positive than on negative selection (~50% increase of the number of $CD4^+$ SP cells compared to 25% decrease of DP cells; Table 1). In the Plas *et al*. study, the loss of SHP-1 activity was generated by expression of a dominant negative mutant of SHP-1 in mice which, according to the authors, showed a 10 and 25% increase in total SHP-1 expression. Therefore, it is possible that their observation that only positive selection was affected might be due a decrease but not a total loss in SHP-1 activity, thereby more mimicking a *me*/+ mouse than a *me/me* mouse.

SHP-1 has an analogous function in B cells, where it sets the threshold for B cell receptor signaling (41). In the *me/me* mouse, this signaling defect leads to a severe disruption of B cell development. In contrast, overall T cell development appears normal in *me/me* mice (20,22–24). It is well established that *src* family kinases play a major role in lymphocyte development (reviewed in 1,2) and our data strongly suggest that SHP-1 exerts its function as a negative regulator of TCR signaling by regulating Lck activity. Another protein phosphatase, CD45, has been shown to be necessary for activation of *src* family kinases (42,43). Interestingly, CD45 knock-out mice (10) display a phenotype almost the converse of the *motheaten* phenotype with a strong defect in thymocyte development and a minor defect in B cell development, the latter detectable only in the sensitized context of B cell receptor transgenic mice (44). Since it has been shown that some of the B cell defects in *motheaten* mice can be rescued by lack of CD45 (45), it is likely that regulation of antigen receptor signaling via the downstream *src* family kinases is determined both by SHP-1 and CD45.

SHP-1-deficient mice exhibit an early involution of their thymus, most notable in *me*^{v}/*me*^{v} mice. Transplantation experiments of normal and *me*^{v}/*me*^{v} bone marrow suggested that a homing defect of the pro-thymocytes was the cause for the early involution observed in *motheaten* mice (46). However, our data suggest an alternative or additional reason. Due to the increased deletion in *me/me* mice, more thymocytes have to flux through the thymus to sustain a normal total thymocyte number. Eventually, this would lead to a depletion of precursors and, if the mouse lives long enough, result in an early involution of the thymus. This is consistent with our observation that there are very few 'typical' DN thymocytes in *me/me*:Tg mice.

Although our data demonstrate a role for SHP-1 in regulating the thymocyte selection process, the precise molecular mechanism by which SHP-1 exerts such regulation is unclear. One potential, previously described binding partner for SHP-1 at the cell surface is CD5 (21,47). Co-cross-linking of CD5 and the TCR–CD3 complex compared to TCR–CD3 alone leads to a decrease in tyrosyl phosphorylation of a number of substrates (47). Moreover, CD5 has been implicated in negative regulation of TCR signaling, based on studies of TCR Tg mice in a CD5-deficient background. Indeed, such CD5-deficient mice show changes in their thymocyte selection profiles (11) that resemble our observations of SHP-1-deficient mice. In this regard, the role of CD5 in T cells might be analogous to that of CD22 in B cell signaling (48–51). Alternatively, it is well documented that SHP-1 becomes tyrosyl phosphorylated rapidly following T cell activation (14). This indicates its proximity to the PTKs that become activated upon TCR engagement and tyrosyl phosphorylation might be a possible means of recruitment. Our observations (20 and the data presented above) strongly suggest that SHP-1 functions upstream of *src* family kinases such as Lck and Fyn. Since Zap-70 is downstream of Lck in the TCR signaling cascade, it is feasible that the previously described regulation of Zap-70 activity by SHP-1 (22) is, in fact, due to the regulation of Lck by SHP-1 with Zap-70 being affected indirectly. Alternatively, both Lck and Zap-70 could be direct targets of SHP-1. Given the previously established role for Lck in regulating TCR-mediated events during thymocyte development, it is possible that SHP-1 may regulate thymocyte selection events by directly controlling activation of Lck. However, additional evidence addressing whether Lck is a direct or a downstream target remains to be supplied. Further studies will be necessary to identify the specific downstream molecule(s) through which SHP-1 exerts its regulation of the TCR signal strength during thymocyte development. Since the selection of an appropriate repertoire of T cells is critical for normal immune responses and for preventing autoimmune dis-

eases and immunodeficiencies, it will be important to identify these targets of SHP-1.

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Abbreviations

APC	antigen-presenting cell
DP	double positive
DN	double negative
GST	glutathione-S-transferase
PTK	protein tyrosine kinase
SH2	Src-homology region 2
SP	single positive
Tg	transgenic

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