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Regulation of T Cell Homeostasis by the Transmembrane Adaptor Protein SIT

Vilmos Posevitz, Boerge Arndt, Tina Krieger, Nicole Warnecke, Burkhart Schraven, and Luca Simeoni

The transmembrane adaptor protein SIT is a negative regulator of TCR-mediated signaling. However, little is known about the functional role of SIT in mature T cells. In this study, we show that mice deficient for SIT display a decreased number of naive CD8+ T cells and a progressive accumulation of memory-like (CD44high) CD8+ T lymphocytes that resemble cells undergoing homeostatic proliferation. Indeed, when transferred into lymphopenic hosts, SIT−/− naive CD8+ T cells undergo enhanced homeostatic proliferation and express a higher level of CD44 in comparison to wild-type T cells. By using class-I-restricted TCR transgenic mice with different ligand affinity/avidity, we show that lymphopenia-induced homeostatic proliferation is more pronounced in cells carrying low-affinity TCRs. Strikingly, the loss of SIT induces homeostatic proliferation of HY TCR transgenic cells, which are normally unable to proliferate in lymphopenic mice. Collectively, these data demonstrate that SIT negatively regulates T cell homeostasis. Finally, we show that SIT-deficient T cells develop a mechanism analogous to sensory adaptation as they up-regulate CD5, down-regulate the coreceptor, and display impaired TCR-mediated ZAP-70 activation.


The number of mature T cells is kept constant throughout the life by a tightly regulated process termed peripheral homeostasis (1). Over the past several years, studies have demonstrated that different mechanisms contribute to the maintenance of the pool of peripheral T lymphocytes. In the days following birth, the periphery is filled with naive cells emigrating from the thymus. Once these naive cells have colonized the periphery, they undergo a cell division program called homeostatic proliferation (2). Both processes, namely emigration from the thymus and expansion in the periphery, determine the initial size of the peripheral T cell pool. Prolonged survival and homeostatic proliferation of peripheral T lymphocytes allow to keep cell numbers constant during adulthood when the thymus involutes and thymic output strongly decreases. Homeostatic proliferation is also necessary to replenish the peripheral T cell pool following abnormal cell depletion caused by e.g., irradiation, chemotherapy, or infection (3–5).

Signals emanating from the TCR as well as from IL receptors (e.g., IL-7, IL-15, among others) critically regulate lymphocyte homeostasis (6–8). Several groups have addressed the role of the TCR in maintaining the peripheral T cell pool. The TCR has the ability “to sense” differences in ligand affinity and in turn is able to translate these different input signals into distinct cellular responses. The most explicit example of this phenomenon is thymic selection, where thymocytes expressing TCRs with high affinity for self-peptide/MHC complexes are negatively selected whereas those expressing TCRs with lower affinity are positively selected and mature into CD4+ or CD8+ cells (9, 10). Experimental evidence also suggests that interaction with self-peptide/MHC serves the additional purpose of maintaining cell survival in the periphery and of triggering homeostatic proliferation of mature T lymphocytes (11, 12). The essential role of signal strength in T cell homeostasis highlights the importance of those molecules that are able to modulate TCR-mediated signaling.

In this regard, adaptor proteins, a group of molecules that organize signaling complexes at the plasma membrane, play a pivotal role as they represent important regulators of lymphocyte homeostasis. Previously, we have shown that Src homology domain containing tyrosine phosphatase 2-interacting transmembrane adaptor protein (SIT),3 a transmembrane adaptor strongly expressed on thymocytes and peripheral T cells, represents a multifunctional regulator of TCR-mediated signaling (13–15). The characterization of SIT-deficient mice demonstrated that SIT regulates selection processes within the thymus by setting TCR-mediated activation thresholds (13). Indeed, positive selection was partially converted to negative selection and the expression level of CD5 was strongly elevated on SIT−/− CD4+ CD8+ immature thymocytes. CD5 is a molecule whose expression is directly proportional to the affinity of the TCR for self-peptide/MHC ligands (16). Collectively, our data had demonstrated that SIT negatively regulates TCR-mediated signal strength. As the maintenance of the peripheral T cell pool is also strictly dependent upon TCR-mediated signaling, we here investigated the role of SIT in peripheral T cell homeostasis. To date, the number of molecules that have been shown to regulate homeostatic proliferation is surprisingly limited and nothing is known about the role that transmembrane adaptor proteins play in this process.

Abbreviations used in this paper: SIT, Src homology domain containing tyrosine phosphatase 2-interacting transmembrane adaptor protein; LAT, linker for activation of T cells; WT, wild type; BTLA, B and T lymphocyte attenuator.

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We report that SIT is required for proper homeostasis of peripheral T cells and negatively regulates homeostatic proliferation in lymphopenic hosts. SIT-deficient T cells further develop sensory adaptation and display impaired ZAP-70 activation. Collectively, the data shown in this study demonstrate that SIT is a critical modulator of the signaling threshold and regulates T cell development and peripheral homeostasis in a similar fashion.

Materials and Methods

Mice
SIT−/− mice were previously described (13). All mice used in this study were between 1 and 5 mo of age, when not otherwise indicated. OT-I TCR transgenic mice were provided by Dr. Percy Knorre, P14 by Dr. Thomas Kammerthoens, and HY TCR transgenic mice by Dr. Gary Koretzky. SIT−/− transgenic mice were provided by Dr. Percy Knolle, P14 by Dr. Thomas Kammerthoens, and HY TCR transgenic mice by Dr. Gary Koretzky. SIT−/− transgenic mice were provided by Dr. Percy Knolle, P14 by Dr. Thomas Kammerthoens, and HY TCR transgenic mice by Dr. Gary Koretzky. All mice used in this study were previously described (13). All mice were subjected to immunoblotting, or immunoprecipitated with Abs specified in the Results section.

Flow cytometry
For FACS analysis, single cell suspensions were prepared and 1 × 10^6 cells were stained with indicated Abs and analyzed on a FACS Calibur using the CellQuest software (Becton Dickinson). All Abs were purchased from BD Biosciences except for HY (clone T3.70; eBioscience) as previously reported (13). For apoptosis determination, splenocytes and lymph node cells were harvested, washed, and stained with Annexin V-PE (BD Biosciences). Intracellular staining
Intracellular staining was performed by using a Bcl-2 FITC kit (BD Biosciences) according to the manufacturer’s instructions.

Brdu labeling and analysis
Steady-state proliferation of T cells was measured by Brdu incorporation. Mice were fed with 0.8 mg/ml BrdU (Sigma-Aldrich) given in their drinking water. Intracellular staining for Brdu was performed by using a FITC Brdu kit (BD Biosciences) in accordance with the manufacturer’s instructions.

T cell isolation
Splenic T cells were purified using a Pan T cell isolation kit (Miltenyi Biotec), while CD4+CD8+ thymocytes were enriched by using anti-FITC microbeads (Miltenyi Biotec) after staining with a FITC-labeled CD8 mAb (clone CT-CD8a, Caltag) on an AutoMACS magnetic separation system. The purity of CD4+CD8+ thymocytes was >97%. CD8 SP thymocytes were enriched by negative selection using CD4 microbeads (Miltenyi Biotec) on an AutoMACS magnetic separation system.

Adoptive transfer
Total lymph node cells or enriched CD8+ SP thymocytes were isolated from mutant and control mice, washed, counted, and suspended at 5 × 10^6 cells/ml in ice cold PBS. Cells were then labeled with 0.1 μM CFSE (Molecular Probes) for 10 min at 37°C. Before i.v. injection, cells were washed with ice cold PBS containing 5% FCS and subsequently resuspended in PBS at 10 × 10^6/ml. Recipient B6.SJL (CD45.1) mice were irradiated (950 rad) using a BioBeam 8000 (STS Steuerungstechnik & Strahlenschutz GmbH) 1 day before adoptive transfer and injected i.v. with 200 μl (2 × 10^6 cells) of cell suspension. After irradiation, mice were treated with 2 mg/ml neomycin sulfate (Sigma-Aldrich). Tissues were harvested on day 3 or 7 and analyzed for the intensity of CFSE and other cell surface markers.

Immunoprecipitation, immunoblotting, in vitro kinase assay
For all biochemical analysis, lymphocytes were either left unstimulated or stimulated with 10 μg/ml biotinylated anti-CD3e Ab (145-2C11; BD Biosciences) alone or in combination with biotinylated CD4 (GK1.5; BD Biosciences) or CD28 (37.51; BD Biosciences) followed by crosslinking with 25 μg/ml streptavidin at 37°C. Cells were lysed in lysis buffer containing 1% Nonidet P-40, 1% laurylmaltoside (N-dodecyl-β-D-maltoside), 50 mM Tris (pH 7.5), 140 mM NaCl, 10 mM EDTA, 10 mM NaF, 1 mM phenylmethylsulfonylfluorid, and 1 mM NaN3. The supernatants of the lysates were subjected to immunoblotting, or immunoprecipitated with Abs specified in the Results section for activation of T cells (LAT) (Transduction Laboratories), ZAP-70 (Santa Cruz Biotecnology), or TCR-ζ (Santa Cruz Biotecnology). Abs were captured using protein A-Sepharose. Proteins were separated by SDS-PAGE, electrotransferred to polyvinylidene difluoride membranes, and blotted with the following Abs: anti-phosphotyrosine (4G10), anti-ERK1/2 (Cell Signaling Technology), anti-JNK (Cell Signaling Technology), anti-p38 (Cell Signaling Technology), anti-phosphoLAT (Y171, Cell Signaling Technology; Y191, Cell Signaling Technology; Y226, Upstate Biotechnology), anti-LAT (a gift from Dr. Weiguo Zhang) anti-phosphoZAP-70 (Y319, Y493, Cell Signaling Technology), and anti-ZAP-70 (Transduction Laboratories).

Ca2+ flux
For Ca2+-measurement, cells were incubated for 30 min with 3.75 μg/ml indo-1-AM (Molecular Probes) in phenol red-free RPMI 1640 medium (Invitrogen Life Technologies) containing 10% FCS. After washing, the cells were incubated in the presence of CD4-FITC, CD8-PE, and 10 μg/ml biotinylated CD3 (145-2C11; BD Biosciences) mAbs at 4°C. Calcium fluxes were induced by crosslinking the TCR/CD3 complex with 10 μg/ml biotinylated anti-CD3 (145-2C11, BD Biosciences) plus 10 μg/ml anti-CD4 Abs (GK1.5, BD Biosciences) and crosslinked with 25 μg/ml streptavidin at 37°C. Anti-ZAP-70 immunocomplexes were assayed for kinase activity as previously described (17).

Statistics
Statistical analysis was performed using GraphPad Prism (GraphPad Software). Mean values ± SEM are shown in each graph. Asterisks represent p values of an unpaired two-tailed Student’s t test.

Results

SIT−/− mice show a reduction of CD44low and an accumulation of CD44high CD8+ T cells

We have previously shown that SIT deficiency resulted in a selective decrease in the numbers of T, but not B cells in lymph nodes. This deficit seems to affect particularly the CD8+ T cell pool (Fig. 1A and Ref. 13). To further elucidate this phenomenon, we investigated the survival rate and steady-state proliferation of peripheral T cell subpopulations. To this end, we firstly measured the expression of Brd-2, an antiapoptotic molecule whose expression is regulated by survival signals (6, 18). Fig. 1B shows that intracellular expression of this protein was not affected in the absence of SIT. Furthermore, the survival rate after in vitro culture (data not shown) or Annexin V expression on ex vivo isolated CD8+ T cells (Fig. 1C) were not altered in the absence of SIT. Hence, it appears as if loss of SIT does not affect the survival of CD8+ as well as CD4+ T cells (data not shown).

We next measured steady-state proliferation by determining BrdU incorporation in CD8+ T cells. However, we found similar turnover rates between SIT−/− and wild-type (WT) mice (Fig. 1D).

Collectively, these data suggest that loss of SIT does not alter T cell numbers by affecting their survival or steady-state proliferation.

To shed further light onto this phenomenon, we characterized the expression of differentiation markers in lymphoid organs of SIT-deficient and WT mice by flow cytometry.

We initially assessed the expression of CD44, a cell surface marker that is generally used to distinguish between naive (CD44low) and memory cells (CD44 high). Fig. 2A shows that CD8+ T cells from SIT−/− mice display a dramatic alteration of the CD44 profile. Calculation of absolute numbers revealed a selective decrease in the numbers of the naive fraction (Fig. 2A). The expression of the activation markers CD25 and CD69 was not altered on T cells from...
SIT-deficient mice (data not shown), thus indicating that the activation status of the cells is normal. These results demonstrate that the paucity of mature CD8⁺T cells observed in the lymph nodes of SIT−/− mice can be exclusively attributed to the loss of the naive CD8 subset. Furthermore, SIT-deficient mice also accumulate high numbers of CD44high CD8⁺T lymphocytes in the spleen (Fig. 2, B–C).

In summary, these data show that SIT regulates the differentiation status of T cells. We have previously shown that SIT−/− mice generate less CD8⁺SP thymocytes (13). In agreement with these data, the number of naive CD8⁺T cells is already severely reduced in young SIT−/− mice in which cells released from the thymus significantly contribute to the peripheral T cell pool (Fig. 3A). Moreover, we also found that the fraction of CD8 cells expressing the αE integrin (CD103) (Fig. 3B) and the chemokine receptor CCR9 (data not shown), which have been shown to correspond to a peripheral naive T cell subset that directly originates from recent thymic emigrants (19, 20), are severely reduced in mice lacking SIT. Thus, a decrease in the generation of thymic precursors represents a mechanism to explain the reduction of naive CD8 T cells in SIT-deficient mice.

We next investigated the mechanisms responsible for the accumulation of CD44high T cells in SIT-deficient mice. CD44high cells are usually referred to as memory cells. However, it appears that they represent a pool of cells with different origins including not only Ag-experienced T lymphocytes but also cells undergoing homeostatic proliferation. Indeed, it has been shown that during homeostatic expansion, naive T cells up-regulate CD44 and persist in the secondary lymphoid organs of adult mice, thus contributing to the CD44high lymphocyte pool (21). Therefore, as CD44high cells do not only represent memory cells, we referred to them as memory-like cells.

**FIGURE 1.** Normal survival and steady-state proliferation of CD8⁺ T cells in SIT−/− mice. A, Lymph node cells were isolated from SIT−/− and SIT+/+ mice and stained with CD4 and CD8 mAbs. Bar graphs indicate mean values ± SEM from seven SIT−/− and nine SIT+/+ mice. *p = 0.0217; **p < 0.0001. B, Intracellular Bcl-2 expression in CD8⁺ T cells from lymph nodes of SIT−/− (filled histogram) and SIT−/− (empty histogram) mice. Isotype control is shown as dotted line. C, Lymph node (LN) cells and splenocytes were stained with Annexin V and CD8 mAb. The mean percentage ± SEM of Annexin V⁺ within CD8⁺ T cells from six SIT−/− and seven SIT−/− mice is shown. D, BrdU incorporation was measured for CD8⁺ T cells in lymph nodes (LN) and spleen. Numbers in quadrants indicate percentages of CD8⁺ T cells. The results shown are representative of three independent experiments.

**FIGURE 2.** Altered distribution of CD8⁺ T cell subsets in the periphery of SIT−/− mice. A, Representative CD44 expression profile within CD4⁺ and CD8⁺ T cells from lymph nodes (LN) and spleen of SIT-deficient and control mice. Absolute numbers of CD8⁺ CD44lo and CD8⁺ CD44hi (B) or CD4⁺ CD44lo and CD4⁺ CD44hi (C) T cells from spleens and lymph nodes (LN) of 5-mo-old SIT knockout and control mice were calculated. Filled squares and triangles represent SIT−/− mice, while empty squares and triangles represent SIT+/+ mice.
Further indicating a memory-like phenotype (Fig. 3C), naive T cells up-regulate CD44 compared with WT control T cells. As it has been shown that CD44 is highly expressed on CD8+(SP) thymocytes from knockout and WT mice before adoptive transfer is comparable. This observation indicates that the loss of SIT does not result in a general up-regulation of CD44 expression. In summary, our data demonstrate that the loss of SIT enhances both the homeostatic proliferation of naive T cells and the generation of memory-like T cells in lymphopenic mice. And, therefore, we believe that a similar mechanism is responsible for the accumulation of memory-like T cells in SIT-deficient mice.

As the TCR affinity is reported to correlate with the ability of T cells to undergo homeostatic proliferation in lymphopenic hosts, we performed adoptive transfer using donor cells derived from three TCR transgenic mouse lines, HY, P14, and OT-I, expressing TCRs with different affinities for self-peptides/MHC (Fig. 4C). This experimental design allowed us, first, to study the behavior of homogeneous naive T cell populations and, second, to grade the potential effects of SIT on TCR-mediated signal strength and in turn on homeostatic proliferation.

Because we had previously shown that signaling via the low-affinity HY TCR is particularly affected in SIT-deficient thymocytes, we performed our initial investigations using the HY TCR transgenic system (13). It has been shown that HY CD8+ T cells prepared from female mice do not undergo homeostatic proliferation in lymphopenic hosts, likely because of too low TCR affinity that is not sufficient to trigger expansion of T cells in the periphery (12, 25). To assess whether loss of SIT affects the homeostatic behavior of HY CD8+ T lymphocytes, CFSE-labeled lymph node cells from WT and SIT−/− female HY transgenic mice were adoptively transferred into irradiated female recipient animals. At day 7 after transfer, spleens were isolated and cells analyzed for the number of divisions based on CFSE content. As expected, WT HY CD8+ T cells did not proliferate in lymphopenic female mice (Fig. 4C). In marked contrast, loss of SIT enabled HY TCR transgenic CD8+ T cells to undergo vigorous homeostatic expansion (Fig. 4C). Similar results were obtained when we performed adoptive transfer in a second lymphopenic model, RAG1−/− mice (data not shown). In both systems, SIT−/−/HY CD8+ T cells underwent at least two cell divisions, thus indicating that the proliferation is not a radiation-induced effect, i.e., triggered by cytokines, which are released upon irradiation (26).

The hypothesis that SIT regulates homeostasis primarily via the TCR is further supported by the fact that the in vitro response of T lymphocytes to recombinant cytokines such as IL-7 and IL-15 (which play an important role in peripheral T cell homeostasis; Refs. 7, 8, 18) was comparable in SIT−/− and WT CD8+ T cells (data not shown).

We next tested whether SIT also exerts a negative regulatory effect on CD8+ T cells carrying the P14 TCR, which possesses higher affinity than the HY TCR. Fig. 4C shows that, similarly to the HY TCR transgenic model, the absence of SIT enhanced the homeostatic proliferation of P14 CD8+ T cells.

Finally, we investigated whether SIT has any regulatory effect on the homeostatic capability of CD8+ T cells expressing a high affinity TCR, such as OT-I. In agreement with previously published data, Fig. 4C shows that OT-I TCR transgenic cells strongly proliferate in lymphopenic mice (25). However, Fig. 4 also demonstrates that the kinetics of homeostatic proliferation were comparable between SIT−/− and SIT−/+ OT-I CD8+ T cells. These data suggest that beyond a certain threshold of affinity for self-peptides/MHC complexes, SIT is dispensable for the regulation of recovered after homeostatic proliferation. In agreement with the enhanced homeostatic expansion, we found that SIT-deficient naive CD8+ T cells also up-regulate CD44 at a higher level than their WT counterparts (Fig. 4B). Fig. 4B also shows that the amount of CD44 expressed on CD8+ SP thymocytes from knockout and WT mice before adoptive transfer is comparable. This observation indicates that the loss of SIT does not result in a general up-regulation of CD44 expression. In summary, our data demonstrate that the loss of SIT enhances both the homeostatic proliferation of naive T cells and the generation of memory-like T cells in lymphopenic mice. And, therefore, we believe that a similar mechanism is responsible for the accumulation of memory-like T cells in SIT-deficient mice.

We focused our further investigations on CD8+ T cells as they appear to be more affected by the loss of SIT than CD4+ T cells. To characterize in more detail CD8+CD44high T cells in SIT-deficient mice, we measured the expression of additional markers of cell differentiation. Fig. 3C shows that CD62L and CD122 were both highly expressed on CD8+CD44high T lymphocytes thus further indicating a memory-like phenotype (Fig. 3C). As CD8+CD44highCD62LhighCD122high lymphocytes resemble cells undergoing homeostatic proliferation (22–24), the data shown in Fig. 3C suggest that homeostatic expansion is enhanced in SIT-deficient mice. To explore this possibility, we performed adoptive transfer experiments.

Loss of SIT lowers the activation threshold and enhances homeostatic proliferation of CD8+ T cells

Adoptive transfer of CFSE-labeled lymph node cells into lymphopenic mice is a well-established experimental system to study homeostatic mechanisms. To investigate whether the loss of SIT resulted in enhanced T cell homeostatic proliferation, we adoptively transferred enriched CD8+ SP thymocytes, as a source of naive T cells, in irradiated recipient mice. Fig. 4A shows that SIT-deficient CD8+ T cells undergo a faster homeostatic proliferation (22–24), the data shown in Fig. 4A demonstrates that the kinetics of homeostatic proliferation naive T cells up-regulate CD44 expression (21), we measured the level of this differentiation marker on CD8+ T cells from SIT-knockout and control mice recovered after homeostatic proliferation. In agreement with the enhanced homeostatic expansion, we found that SIT-deficient naive CD8+ T cells also up-regulate CD44 at a higher level than their WT counterparts (Fig. 4B). Fig. 4B also shows that the amount of CD44 expressed on CD8+ SP thymocytes from knockout and WT mice before adoptive transfer is comparable. This observation indicates that the loss of SIT does not result in a general up-regulation of CD44 expression. In summary, our data demonstrate that the loss of SIT enhances both the homeostatic proliferation of naive T cells and the generation of memory-like T cells in lymphopenic mice. And, therefore, we believe that a similar mechanism is responsible for the accumulation of memory-like T cells in SIT-deficient mice.

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TCR-mediated signaling. Collectively, these data demonstrate that SIT-deficiency lowers TCR-mediated activation threshold and enhances homeostatic proliferation.

Sensory adaptation of SIT-deficient T cells

Despite the lower threshold for activation, CD8⁺ T cells from SIT-deficient mice do not display a fully activated phenotype. Therefore, it is conceivable that SIT⁻/⁻ cells develop compensatory mechanisms to avoid improper activation. Indeed, recent observations suggest that T cells are endowed with adaptation mechanisms and may reprogram the activation threshold in reaction to strong signals (27). To assess this point, we investigated the expression of CD5, which is well established to directly correlate with the strength of TCR engagement. Moreover, the expression of CD5 is required to dampen strong TCR signaling (28). CD5 function appears to be of particular importance in CD4⁻CD8⁺ thymocytes, whose maturation critically depends upon the TCR-mediated activation threshold. In fact, we and others have previously shown that thymocytes lacking negative regulatory molecules express higher levels of CD5 as compared with WT cells (13, 29, 30). Surprisingly, we found that CD5 expression remained elevated even on peripheral SIT⁻/⁻
in the presence of persistent stimulation. Modulation of T cell responsiveness to regulate the threshold of T cell activation (31). Thus in addition to upregulating CD5, modulation of T cell responsiveness of SIT-deficient T cells was further supported by the observation that thymocytes and peripheral T cells displayed impaired proximal signaling events upon in vitro stimulation with CD3 or CD3 plus CD28 Abs (Fig. 6A and our unpublished data). The data depicted in Fig. 6A show that tyrosine phosphorylation of several proteins is reduced in SIT-deficient thymocytes. In particular, we consistently observed a marked reduction of TCR-mediated tyrosine phosphorylation of LAT either assessed by global anti-phosphotyrosine immunoblotting (Fig. 6A), LAT immunoprecipitation, or by using phosphosite specific Abs (Fig. 6B).

Given the strong reduction in LAT phosphorylation it was necessary to investigate whether the activation of signaling molecules downstream of LAT is also reduced in SIT-deficient thymocytes. Fig. 6A shows that TCR-mediated activation of ERK1/2 is significantly impaired in SIT-deficient mice whereas the activations of JNK/SAPK and p38 are normal. In agreement with these data, further biochemical analyses revealed that TCR-mediated phosphorylation of PLCγ1 and calcium-flux are reduced in SIT-deficient T cells (Fig. 6C).

ZAP-70 is believed to represent the major tyrosine kinase responsible for LAT phosphorylation (32). To assess whether the reduced levels of LAT phosphorylation in SIT-deficient mice is due to an impaired activation of ZAP-70 we investigated the phosphorylation status and the enzymatic activity of ZAP-70. In line with blunted LAT phosphorylation we found that TCR-mediated phosphorylation of two critical tyrosine residues of ZAP70, Y319, and Y493 is reduced in SIT-deficient thymocytes (Fig. 6C). Similary, in vitro kinase assays of ZAP-70 immunoprecipitates showed attenuated enzymatic activity of ZAP 70 in the absence of SIT (Fig. 6D).

ZAP-70 activation requires the tyrosine phosphorylation of ITAM motifs of the CD3 and TCR-ζ-chains (33). Phosphorylation of the ITAMs provides docking sites for the two SH2 domains within ZAP-70, thus positioning this kinase in the TCR/CD3 complex closed to its upstream activator Lck. Therefore, to elucidate the mechanisms responsible for impaired ZAP-70 activation in SIT-deficient T cells, we investigated TCR-ζ phosphorylation and TCR-ζ/ZAP-70 association. Fig. 6E shows that complete tyrosine phosphorylation of TCR-ζ upon CD3-crosslinking, which is detectable as a phospho-protein migrating at 23kDa, is comparable between SIT-deficient and WT T cells (Fig. 6E). Furthermore, analysis of immunoprecipitated TCR-ζ show that the association with ZAP-70 is also normal in SIT–/– T cells (Fig. 6E), thus suggesting that the phosphorylation of TCR-ζ and the recruitment of ZAP-70 to the TCR/CD3 complex are intact in SIT-deficient mice. Overall, our findings indicate the existence of a molecular mechanism that renders SIT-deficient T cells refractory to TCR-mediated stimulation by reducing ZAP-70 activation.

CD8+ T cells either in TCR transgenic or nontransgenic genetic backgrounds (Fig. 5A and our unpublished data). This observation corroborates the hypothesis that in addition to thymocytes, peripheral CD8+ T lymphocytes receive constantly stronger signals via the TCR in the absence of SIT.

To further characterize the existence of mechanisms that would allow tuning of the activation threshold in SIT–/– mice, we investigated the expression of the coreceptor CD8. Recent data suggested that down-regulation of CD8 is a mechanism that allows T cells to regulate the threshold of T cell activation (31). Thus in addition to upregulating CD5, modulation of T cell responsiveness by downregulating the CD8 coreceptor represents a mechanism for sensory adaptation that prevents the activation of autoreactive T cells in the presence of persistent stimulation.

Analysis of SIT–/– peripheral CD8+ T cells revealed significantly lower levels of CD8 expression compared with control mice (Fig. 5A). As CD8+ single positive thymocytes express normal level of CD8 (data not shown), the down-regulation of the coreceptor in the periphery is likely induced by signals received by mature T cells. A similar coreceptor down-regulation was detected in SIT-deficient mice carrying the HY and P14 TCRs (data not shown). Moreover, HY and P14 SIT–/– mice also displayed unusually high numbers of transgenic TCRαCD4+CD8+ lymphocytes in the periphery (Fig. 5B), thus likely indicating that a complete down-regulation of the coreceptor has occurred in some cells.

In addition to CD8+ T cells, SIT-deficient CD4+CD8+ thymocytes show a similar phenotype and up-regulate CD5 while down-regulating CD4 (data not shown). Taken together, these observations are in line with the concept that SIT-deficient T cells counterbalance the stronger TCR-mediated stimulation by adjusting the expression of positive and negative regulatory molecules, thus reprogramming the activation threshold.

**Impaired proximal signaling in SIT–/– T cells**

The existence of compensatory mechanisms in SIT-deficient mice was further supported by the observation that thymocytes and peripheral T cells displayed impaired proximal signaling events upon in vitro stimulation with CD3 or CD3 plus CD28 Abs (Fig. 6A and our unpublished data). The data depicted in Fig. 6A show that tyrosine phosphorylation of several proteins is reduced in SIT-deficient thymocytes. In particular, we consistently observed a marked reduction of TCR-mediated tyrosine phosphorylation of LAT either assessed by global anti-phosphotyrosine immunoblotting (Fig. 6A), LAT immunoprecipitation, or by using phosphosite specific Abs (Fig. 6B).

Given the strong reduction in LAT phosphorylation it was necessary to investigate whether the activation of signaling molecules downstream of LAT is also reduced in SIT–/– thymocytes. Fig. 6A shows that TCR-mediated activation of ERK1/2 is significantly impaired in SIT-deficient mice whereas the activations of JNK/SAPK and p38 are normal. In agreement with these data, further biochemical analyses revealed that TCR-mediated phosphorylation of PLCγ1 and calcium-flux are reduced in SIT-deficient T cells (Fig. 6C).

ZAP-70 is believed to represent the major tyrosine kinase responsible for LAT phosphorylation (32). To assess whether the reduced levels of LAT phosphorylation in SIT-deficient mice is due to an impaired activation of ZAP-70 we investigated the phosphorylation status and the enzymatic activity of ZAP-70. In line with blunted LAT phosphorylation we found that TCR-mediated phosphorylation of two critical tyrosine residues of ZAP70, Y319, and Y493 is reduced in SIT-deficient thymocytes (Fig. 6C). Similary, in vitro kinase assays of ZAP-70 immunoprecipitates showed attenuated enzymatic activity of ZAP 70 in the absence of SIT (Fig. 6D).

ZAP-70 activation requires the tyrosine phosphorylation of ITAM motifs of the CD3 and TCR-ζ-chains (33). Phosphorylation of the ITAMs provides docking sites for the two SH2 domains within ZAP-70, thus positioning this kinase in the TCR/CD3 complex closed to its upstream activator Lck. Therefore, to elucidate the mechanisms responsible for impaired ZAP-70 activation in SIT-deficient T cells, we investigated TCR-ζ phosphorylation and TCR-ζ/ZAP-70 association. Fig. 6E shows that complete tyrosine phosphorylation of TCR-ζ upon CD3-crosslinking, which is detectable as a phospho-protein migrating at 23kDa, is comparable between SIT-deficient and WT T cells (Fig. 6E). Furthermore, analysis of immunoprecipitated TCR-ζ show that the association with ZAP-70 is also normal in SIT–/– T cells (Fig. 6E), thus suggesting that the phosphorylation of TCR-ζ and the recruitment of ZAP-70 to the TCR/CD3 complex are intact in SIT-deficient mice. Overall, our findings indicate the existence of a molecular mechanism that renders SIT-deficient T cells refractory to TCR-mediated stimulation by reducing ZAP-70 activation.
Discussion
The data presented in this study reveal that SIT, a nonraft-associated transmembrane adaptor, acts as a critical regulator of the peripheral T cell pool. We demonstrate that SIT contributes to the maintenance of the peripheral CD8\(^+\) T cell in at least two different ways. First, by regulating the number of precursors generated within the thymus and, second, by modulating homeostatic proliferation in the periphery. Our data suggest that SIT lowers the T cell-activation threshold whereby signals that normally should promote survival may become overtly stimulatory in the absence of
SIT and may induce naive CD8+ and CD4+ lymphocytes to undergo a continuous slow-rate homeostatic proliferation. During this expansion, naive cells seem to progressively acquire the memory-like phenotype.

Despite the fact that SIT is expressed in both cell types, SIT-deficiency more prominently affects CD8+ than CD4+ T cells. Similarly, mice lacking the adaptor protein GADS, which, like SIT, is expressed in both T-lymphocyte subsets, show an impaired homeostasis in CD4+ but not in CD8+ T cells (34). These results are in line with the idea that homeostasis in CD8+ T cells is regulated differentially than in CD4+ T cells.

We propose that SIT represents a modulator of T cell homeostasis. Conversely to other molecules such as LAT or CTLA-4 that have been shown to regulate the expansion of activated T cells (35–38) SIT and the recently published human lymphocyte activation Ag 3 (39) and B and T lymphocyte attenuator (BTLA) (40) appear to be the only negative regulatory molecules known so far that selectively affect peripheral T cell homeostasis without altering the activation status of T cells.

One mechanism of action could be that SIT recruits tyrosine phosphatases such as SHP-1 and SHP-2 to the plasma membrane (41) are enhanced in SIT-deficient T cells. Alternatively, tyrosine phosphatases such as SHP-1, which are known to mediate dephosphorylation and inactivation of ZAP-70 (50) are more active in SIT-deficient T cells. In agreement with this hypothesis would be our finding that the expression of inhibitory receptors such as CD5, a molecule that down-regulates the TCR-mediated signal transduction pathway by recruiting SHP-1 to the plasma membrane (41) are enhanced in SIT-deficient T lymphocytes.

Survival and homeostatic proliferation of peripheral T cells both require the expression of self-peptide/MHC molecules (47). This requirement parallels the need for MHC restriction during Ag-specific proliferation. However, unlike Ag-induced proliferation, homeostatic proliferation does not require co-stimulation (23, 48) and does not result in the differentiation of effector cells (11, 23). Compelling evidences further suggest that homeostatic expansion of peripheral T cells has similarities to T cell development. Indeed, two groups have recently shown that the peptides initiating positive selection in the thymus also drive homeostatic proliferation in the periphery (11, 12). Therefore, self peptides appear to be key factors for determining cell fate. Upon TCR ligation, they rescue T lymphocytes from a default cell death pathway, thus initiating positive selection in the thymus, and, further, they induce homeostatic proliferation in the periphery. Consequently, TCR-ligand affinity plays a pivotal role in the regulation of the efficiency of these processes. In fact, transgenic T cells carrying the low affinity HY TCR undergo inefficient positive selection and show no homeostatic proliferation, whereas mice expressing TCRs with higher affinities, such as P14, 2C, and OT-I, show a more efficient positive selection and robustly proliferate in lymphopenic mice (25, 49). Thus, it is likely that thymic selection and homeostatic proliferation share similar regulatory mechanisms. How a T cell senses the degrees of TCR-ligand affinity and sorts them into specific cellular outcomes is largely unknown. However, the data presented in this study as well as our previous study on SIT-deficient mice (13) demonstrate that SIT is an important component of the signaling machinery that translates analog inputs into digital outputs and suggest that SIT functions as a fine-tuner of the translation process. To our knowledge, SIT is the only molecule that has been shown so far to regulate both thymic development and homeostatic proliferation.

Our data suggest that SIT regulates T cell homeostasis primarily via the TCR. However, we cannot exclude the possibility that in vivo SIT could also regulate signal transduction from other receptors such as IL-2 receptors or even a cross-talk between the TCR and IL-1 receptors.

We also show that SIT-deficient T cells develop sensory adaptation, most likely in an attempt to compensate the enhanced TCR-mediated signaling. At the molecular level, SIT−/− T cells show an impaired ZAP-70 activity that in turn affects the activation of other downstream signaling molecules. Therefore, the perturbation of ZAP-70 activity is the most apical defect in the TCR-mediated pathway that is affected by SIT. How does loss of SIT impair ZAP-70 activation? We propose two mechanisms to answer this question. First, as SIT-deficient T cells down-regulate coreceptor expression, the amount of Lck that is activated upon TCR/coreceptor coengagement is reduced. The defective Lck activation could in turn result in a selective reduction of ZAP-70 activation. As we did not notice changes in TCR-ζ phosphorylation, it is likely that src-kinase activity associated with the TCR is not affected in SIT-deficient T cells. Alternatively, tyrosine phosphatases such as SHP-1, which are known to mediate dephosphorylation and inactivation of ZAP-70 (50) are more active in SIT-deficient T cells. In agreement with this hypothesis would be our finding that the expression of inhibitory receptors such as CD5, a molecule that down-regulates the TCR-mediated signal transduction pathway by recruiting SHP-1 to the plasma membrane (41) are enhanced in SIT-deficient T lymphocytes.

The development of compensatory mechanisms could serve to prevent autoimmune or chronic inflammatory processes and hence to preserve an appropriate immune competence of T cells even in the absence of SIT. Indeed, immune responses against Ags, Toxoplasma, and Listeria, as well as rejection of allograft transplantations were all normal in SIT-deficient mice (our unpublished observations). However, despite the apparently normal immune response, it appears as if the sensory adaptation is not completely effective. In fact, we have recently shown that SIT-deficient mice spontaneously develop antinuclear Abs, glomerulonephritis, and accumulate activated CD4 T cells in secondary lymphoid organs (our unpublished results). Additionally, we showed previously that SIT-deficient mice display a more severe clinical course during Experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis (13). Individuals in which T cells undergo enhanced lymphopenia-induced homeostatic proliferation such as patients affected by immunodeficiencies (i.e., AIDS) or treated with chemo/radio therapy suffer from different autoimmune diseases (51, 52). Moreover, it has been demonstrated that T cells undergoing homeostatic expansion are more susceptible to becoming autoreactive (53). On the basis of these observations, we propose that the susceptibility of SIT−/− mice to develop autoimmunity is likely caused by the enhanced homeostatic proliferation of SIT-deficient T cells. In conclusion, our observations suggest that SIT plays an important role in maintaining T cell homeostasis and that alterations of SIT expression or function in humans may lead to enhanced homeostatic proliferation and to an increased susceptibility to develop autoimmune diseases.

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Disclosures

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