The transmembrane adaptor protein SIT regulates T-cell development and homeostasis

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Abstract

In this study, I characterised mice lacking the transmembrane adaptor protein SIT. I found that the loss of SIT resulted in altered T-cell development. Indeed, thymocytes deficient for SIT displayed a more activated phenotype and an enhanced positive selection that was even partially converted to negative selection in HY and P14 TCR transgenic mice. However, $SIT^{-/-}$ mice expressing a TCR with high affinity, such as OT-I, did not show any alteration of T-cell development. This indicates that the ability of SIT to regulate thymic selection processes is dependent on TCR affinity. Moreover, SIT knockout mice displayed a decreased number of SP thymocytes as well as a decreased proportion of recent thymic emigrants in the periphery. In addition, further investigation revealed that $SIT^{-/-}$ mice have strongly reduced number of naïve $CD8^+$ T cells. We showed that the loss of naïve $CD8^+$ T cells is neither due to a survival defect nor to enhanced apoptosis but it is partially caused by a reduction in the generation of T-cell precursors in the thymus. Moreover, SIT-deficient mice progressively accumulate $CD44^{hi}CD8^+$ cells in the periphery. A more detailed characterisation of those cells revealed that they closely resemble lymphocytes undergoing homeostatic proliferation. To directly test the role of SIT in T-cell homeostasis, I performed adoptive transfer experiments by injecting CFSE labelled lymph node cells from MHC-I restricted transgenic mice with low (HY), intermediate (P14) and with high (OT-I) TCR affinity into lymphopenic hosts. Strikingly, loss of SIT results in homeostatic expansion of HY transgenic T cells that normally do not undergo homeostatic proliferation in lymphopenic hosts. Similarly, $SIT^{-/-}$P14 T cells showed a more accelerated homeostatic expansion upon adoptive transfer into lymphopenic recipients as compared to $SIT^{+/+}$P14 cells. Similar to T-cell development, lymphopenia-induced proliferation of OT-I $CD8^+$ T cells was not affected by the loss of SIT. These results clearly indicate that SIT is a potent negative regulator of both T-cell development and homeostasis. Our data also demonstrate that the reduced number of naïve $CD8^+$ T cells in SIT-deficient mice is likely due to an accelerated homeostatic expansion. During this process, $CD8^+$ T cells acquire $CD44^{hi}$ expression. In addition to defective T-cell development and homeostasis, $SIT^{-/-}$ T cells are hyperreactive to TCR-mediated stimuli *in vitro* and develop more severe EAE. Collectively, these observations clearly indicate that loss of SIT results in a lowered threshold of activation in both thymocytes and T cells. Despite the fact that SIT-deficient mice displayed a perturbed T-cell homeostasis, loss of SIT did not result in any obvious sign of altered immune function in young mice. Thus, we

hypothesised a presence of a compensatory mechanism(s) that would prevent SIT deficient T cells from becoming fully activated.

Indeed, I showed that $SIT^{-/-}$ $CD8^{+}$ T cells develop sensory adaptation by adjusting coreceptor and CD5 expression. Moreover, additional data from our laboratory showed that in vitro Zap-70 activity is severely impaired in SIT deficient T cells. It is likely that compensatory mechanism(s) present in $SIT^{-/-}$ T cells is (are) sufficient to prevent T cells from becoming fully activated. However, this mechanism fails to prevent altered T-cell development, lowered threshold of activation as well as altered peripheral T-cell homeostasis.

Collectively, I showed that SIT acts as a negative regulator of T-cell development and homeostasis by modulating TCR signalling threshold.

Reagents and recipes

PCR reagents

Taq Polymerase (Qiagen)
Deoxynucleotide (dNTP) Solution Mix (Qiagen)
Enhancer Q solution (Qiagen)
$MgCl_2$ (Qiagen)
10xTaq Buffer (Qiagen)
DMSO (dimethyl-sulfoxide, Sigma)

Agarose gel (2%)

2g agarose (PeqLab)
100ml 1xTAE buffer
10µl etidium bromide (Roth)

50xTAE buffer (1l)

242g Tris base (Roth)
57.1ml acetic acid (Baker)
100ml 0.5M EDTA (Sigma) pH 8.0

DNA 6x loading buffer, blue:

0.2g bromphenol blue (Roth)
0.2g xylencyanol
1ml EDTA (500mM)
6ml glycerin (87%)
2ml distilled H_2O (dH_2O)

Cell culture/Mouse Medium:

RPMI 1640 (Biochrom) supplemented with:
10% heat deactivated FCS (Fetal Bovine Serum, PAN Biotech)
2-β mercaptoethanol (50µM) (Sigma)
Antibiotics: Penicillin/Streptamycin (Roth)

Lysis buffer

1% NP-40 (Nonidet P-40, Igepal, Sigma)
1% LM (laurylmaltoside) (Calbiochem)
1mM sodium monovanadate (Merck)
1mM PMSF (phenilmethylsulfonyl fluoride, Sigma)
50mM Tris (ph:7.5) (Roth)
10mM NaF (Sigma)

10mM EDTA (ph 7.5) (Sigma)
166mM NaCl (Roth)
657µl (dH_2O)

NP-40 washing buffer

1% 0.5M EDTA (Sigma)
5% 1M Tris pH7.4 (Roth)
5% NP-40 (Igepal, Sigma)
15% 1M NaCl (Roth)

5x reducing sample-buffer (for 10 ml)

5ml glycerol (Sigma)
2ml 0.5M Tris, pH 6.8 (Roth)
2.5 ml 20% SDS (Calbiochem)
0.25ml 10% bromphenol blue (Roth)
50µl 2-β-mercaptoethanol (Sigma)

Gel electrophoresis and Western blot

SDS-PAGE gel (for 5 ml, 10% separating gel)
2.1ml (dH_2O)
1.6ml 30% acrylamide/BIS (Bio-Rad)
1.23 ml 1.5M Tris-HCl pH8.8 (Roth)
49.2µl 10% SDS (Calbiochem)
24.6µl 10% APS (Ammoniumperoxidisulphate, Roth)
2.46µl TEMED (Roth)

SDS-PAGE gel (for 2 ml stacking gel)

1.2ml (dH_2O)
0.375ml 40% acrylamide/BIS 29:1 (Bio-Rad)
0.5ml 0.5M Tris-HCl pH6.8 (Roth)
20µl 10% SDS (Calbiochem)
20µl 10% APS (Roth)
2µl TEMED (Roth)

Transblot solution (for 1l)

5.80g Tris (Roth)
2.90g glycine (Roth)
0.37g SDS (Calbiochem)
200ml methanol (Roth)

10xTBS buffer (for 1l)

80.0g NaCl (Roth)
2.0g KCl (Sigma)
30.0g Tris (Roth) pH 8.00

Stripping buffer (for 200ml)

40.0ml 10% SDS (Calbiochem)
1.4ml 2-β-mercaptoethanol (Sigma)
25.0ml 0.5M Tris, pH 6.7 (Roth)

Others

10xTGS - electrophoresis buffer for SDS-PAGE gel (Bio-Rad)
Tween-20 (Roth)
Protein MW standard: All Blue (BioRad)
DNA: PeqGold Leiter mix (Peqlab)
ECL Kit (Amersham)
PBS Dulbecco's (Gibco)
BSA (albumine, bovine serum, fraction V, Sigma)

Materials and methods

Mice

The targeting construct to inactivate *sit* gene was generated by Dr. Eddy Bruyns. SIT knockout mice were generated by Prof. Klaus Pfeffer as previously described (4). Animals containing the disrupted SIT allele were backcrossed onto C57BL/6 (Charles River) for more than 10 generations. OT-I and OT-II TCR transgenic mice were kindly provided by Dr. Percy Knolle, P14 by Dr. Thomas Kammerthoens, and HY TCR transgenic mice were kindly provided by Dr. Gary Koretzky. For TCR transgenic studies, $SIT^{-/-}$ mice $\times$ TCR tg mice were obtained by crossing $SIT^{-/-}$ mice with $SIT^{+/-}$ TCR heterozygote transgenic mice. B6.SJL $CD45.1^{+}$ recipient mice for adoptive transfer experiments were kindly provided by Dr. Dirk Schlüter.

Mice genotyping

Genotypes of mice were assessed by standard PCR. Total genomic DNA was purified from mouse tails by using DNeasy Tissue Kit (Qiagen Cat. Nr. : 69506). For PCR reactions, (for details see Appendix) primers were obtained from BioTeZ Berlin-Buch GmbH).

Primers used for genotyping

SIT primers 5´>>3´: primer 1 (CCT GAC TCT CAC ACC AGC AGC), primer 2 (GGT CCA CTG GGA CAA GAG TGC AGC C), primer 3 (GAC GTG CTA CTT CCA TTT GTC ACG TCC), HY primers 5´>>3´: pimer 1 (GCT TTG AGG CCG AGT TTA GG), primer 2 (GCT CAC TGT CAG CTT TGT CC), OT-II primers 5´>>3´: primer 1 (AAA GGG AGA AAA AGC TCT CC), primer 2 (ACA CAG CAG GTT CTG GGT TC), primer 3 (GCT GCT GCA CAG ACC TAC T), primer 4 (CAG CTC ACC TAA CAC GAG GA), OT-I primers 5´>>3´: primer 1 (TTG AGA GCT GTC TCC), primer 2 (AAG GTG GAG AGA GAC AAA GGA TTC), primer 3 (GTC AGT CGA GTG CAC AGT TT) primer 4 (CCA ATG TTG CTT GTC TGG TG), P14 primers 5´>>3´: primer 1 (CTG ACC TGC AGT TAT GAG GAC AGC AC), primer 2 (CGA GGA TCC TTT AAC TGG TAC ACA GCA GG) Rag1 primers 5´>>3´: primer 1 (TGG ATG TGG AAT GTG TGC GAG), primer 2 (GAG GTT CCG CTA CGA CTC TG), primer 3 (CCG GAC AAG TTT TTC ATC GT).

Single cell suspension

Lymphoid organs (thymus, lymph nodes or spleen) were removed from sacrificed wild-type and SIT deficient mice and placed into Petri dishes filled with PBS. Subsequently organs were mechanically disrupted and filtered through nylon mesh with 70-µm pore size. Finally, cells were washed in PBS (1500rpm, 4°C, 10 min) and resuspended either in medium or in PBS. When it was required, all steps were performed under laminar flow to maintain sterile conditions.

T-cell purification

For standard T-cell purification, spleens from sacrificed mice were removed and single cell suspensions were prepared. T-cell purification was carried out by using Mouse Pan T cell Isolation Kit (Miltenyi Biotech, Order Nr.: 130-090-861) according to the manufacturer's instructions. Purity of cell preparations was assessed by flow cytometry (CD3 and TCR-β stainings) and was routinely >95%.

Proliferation assay and cytokine determination

Purified T cells (2.5×10^4 cells/well) were cultured in RPMI medium (supplemented with 10% FCS, antibiotics and 2-β-mercaptoethanol) in U-bottomed 96 well plate (Costar) at 37°C, 5%CO_2 in a humidified atmosphere, in the presence of plate bound anti-mouse CD3ε (clone: 145-2C11, BD Biosciences) or PMA and ionomycin. Cells were cultured for 72 hours and labelled with 0.5 µCi/well [^{3}H]-thymidine during the last six hours of culture. Supernatants from anti-CD3 stimulated T cells were collected after 48 hours and cytokine level was assessed by cytometric bead array (CBA, BD Biosciences).

Irradiation and adoptive transfer

Single cell suspensions were prepared from lymph nodes of donor mice. Cells were washed (1500rpm, 4°C, 10 min) in PBS, counted and resuspended in ice cold PBS at $5x10^6$ cells/ml. CFSE (Sigma) was added to the cells at 0.2µM final concentration. Samples were incubated at 37°C for 10 min then, washed twice in ice cold PBS supplemented with 5% FCS (1500rpm, 4°C, 10 min). Subsequently cells were resuspended in PBS for intravenous injection at $10x10^6$/ml concentration. Adoptive transfer was performed by injecting 200µl ($2x10^6$ cells) of cell suspension into the lateral tail vein of B6.SJL CD45.1$^+$ recipient mice. Recipient mice were irradiated (dosage: 950rad) one day before adoptive transfer by using BioBeam 8000 (STS

Steuerungstechnik & Strahlenschutz GmbH, 38110 Braunschweig, Germany). During the entire experiment recipient mice were kept on 2mg/ml neomycin sulphate (Sigma, Cat. Nr.: N6386-25G) supplied in the drinking water. Mice were analyzed at day 3 or day 7 after adoptive transfer.

In vitro cytokine stimulation

Lymph node cells were loaded with CFSE (as described above) and washed twice in PBS. Pellets were resuspended at $5x10^6$/ml concentration in mouse medium (for details see reagents and recepies) and cultured in 48-well plates (Costar) at $2x10^6$/ml concentration. Cultured cells were either treated with recombinant mouse IL-7 or IL-15 (R&D Biosystems GmbH) at 100ng/ml final concentration or left untreated. After 72 hours of incubation at 37°C, in a humidified 5% CO_2 atmosphere cells were harvested and subsequently stained for with CD4 and CD8 antibodies. CFSE profiles of gated $CD8^+$ cells were analysed by flow cytometry.

Flow Cytometry

For FACS analyses single cell suspensions were prepared and typically 1×10^6 cells were stained with the indicated antibodies and subsequently analysed on a FACS Calibur using CellQuest software (Becton Dickinson). All antibodies were purchased from BD Biosciences (for more information about antibodies used in this study see Appendix-I). For homeostatic proliferation experiments, single cell suspensions of lymph node and spleen cells from recipient mice were prepared and $1\text{-}3x10^6$ cells were stained with the indicated antibodies.

Intracellular Bcl-2 FACS staining

Lymph nodes were isolated from wild-type and SIT-knockout mice and single cell suspensions were prepared. Subsequently, $1.5x10^6$ cells were stained with CD8-PE antibody. Cell fixation and permeabilization was performed by using Fix. and Perm., solutions from Invitrogen (Cat. Nr.: GAS-004). Bcl-2 intracellular staining was carried out by using Bcl-2-FITC Antibody Reagent Set (BD Biosciences Cat. Nr: 554221) according to the manufacturer`s instructions.

BrdU administration and staining protocol

BrdU (Sigma) was administrated in sterile drinking water at 0.8mg/ml for 7 days and was supplemented with 2% glucose. BrdU solution was prepared daily. Following BrdU administration, wild-type and SIT-knockout mice were sacrificed and single cell suspensions were prepared from lymph nodes and spleens as described previously.

Typically, $3x10^6$ cells were stained with CD4 and CD8 antibodies. Subsequently, intracellular staining to determine BrdU incorporation was carried out by using BrdU Flow Kit (BD Biosciences) according to the manufacturer`s instructions.

Statistics

Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA). Asterisks represent $p<<0.05$ values calculated by unpaired two-tailed Student´s t test.

1. Introduction

1.1. The immune system

Living organisms make an “endless” number of interactions with their environment during the course of their life. These interactions serve as the basis for survival and reproduction, and are therefore indispensable. On the other hand, there are number of dangerous agents such as parasites, bacteria and viruses from which an organism must defend itself to maintain its integrity. Our immune system protects us from pathogens and is ready to prevent any infections that might have serious or life-threatening consequences. A very important requirement that enables the immune system to function is the ability to recognise and differentiate non-self as potentially dangerous from self that is harmless. Proper immune function requires a magnificent and tightly controlled orchestra of numerous participants. In vertebrates, the immune system can be divided into two main components: innate and adaptive immunity. Phylogenetics show adaptive immunity evolved more recently when compared with innate immunity. The cooperation between innate and adaptive immunity results in a well controlled immune response. The components of innate immunity are macrophages, dentritic cells, NK cells, and the complement system. Innate immunity is characterised by an immediate reaction, lack of specificity and the absence of immunological memory. In contrast to the innate immune system, the adaptive immune system possesses some more distinctive functions such as immunological memory and specificity. These features allow adaptive immunity to improve its protective function following recurrent infections. These advanced functions of adaptive immunity are provided by two main cell types namely, T and B lymphocytes. During immune response, innate immunity represents the first line of defense and provides an alarm signal to the adaptive immune system to induce a highly specific and more effective immune response. Here I am going to introduce those components of the adaptive immune system that are of relevance for better understanding my work (1, 46).

1.2. T lymphocytes (Thymus-derived lymphocytes)

1.2.1. Early T-cell development

During embryonic life, all haematopoietic cells are generated in the embryonic spleen and liver. At later developmental stages, lymphoid progenitor cells are generated in the bone marrow. From the bone marrow, progenitor cells migrate via the blood stream to the thymus to complete their maturation and become T lymphocytes. Once they are in the thymus, they become irreversibly committed toward the T-cell lineage. This process is largely dependent on Notch1 receptor signalling (89). Further development of T cells occurs in an ordered progression (summarized in Figure 1) that can be divided into discrete developmental stages defined by the expression of the CD4 and CD8 costimulatory molecules. Most immature T-lymphocyte precursors do not express either CD4 or CD8 and are therefore called DN (double negative $CD4^-CD8^-$) thymocytes. These cells further mature into the DP (double positive) stage ($CD4^+CD8^+$) as they express both CD4 and CD8. At the DP stage, thymocytes undergo a selection process. At the end of the selection process, only less than 5% complete their maturation as $CD4^+$ or $CD8^+$ T cell. Double negative thymocytes are heterogeneous and can be further divided into more distinct subsets that are defined by the expression of CD44 and CD25 (IL2R alpha): DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$), DN4 ($CD44^-CD25^-$) (34). Many efforts have been made to characterise more precisely DN thymocytes. These studies have revealed that DN1 and DN2 thymocytes do not express the CD3 complex and are $CD4^-CD8^-CD3^-$. Thus, they are often termed as triple negative (TN) cells. TN thymocytes express high levels of the receptor for stem cell-factor CD117/c-kit (hereafter referred to as CD117). Thymocytes that express high level of CD117 have a lymphoid restricted potential however, they are still capable of differentiating into B cells and NK cells as well as cells of the T lineage (16, 67). During the transition to the DN3 stage, DN2 thymocytes start to express T-cell specific components such as CD3 and in turn become $CD117^{low}$. At this stage, they are fully committed toward the T-cell lineage. Mice deficient for CD3ε show a developmental arrest at the DN3 stage indicating the essential role of the CD3 complex to regulate further maturation of DN3 thymocytes (60). In addition to the CD3ε, the proper rearrangement of the β chain of the TCR is required for further maturation. During transition from the DN3 to the DN4 stage an essential step named “β-selection” takes pace that in turn inhibits further DNA recombination at the TCRβ locus (allelic exclusion) (119). Allelic exclusion is induced by the successfully rearranged TCRβ chain that prevents any

additional rearrangement at this site, thus, ensuring that only one kind of βTCR is expressed on the surface of each T cell.

Thymocytes undergoing DN3>>DN4 transition do not express a functional αβTCR but instead they express a complex called pre-TCR. Similar to the TCR the pre-TCR coplex includes CD3εγ and εδ heterodimers as well as TCRζζ homodimer and a fully rearranged TCR β chain. Nonetheless, the pre-TCR does not contain the TCRα chain but a surrogate chain called pTα. No ligand for the pre-TCR has been identified so far. Thus, it has been suggested that the pre-TCR is able to signal in an MHC-independent manner. Thymocytes expressing the pre-TCR complex also become CD117 negative indicating that they are fully committed to the T-cell fate (16). In addition, the pre-TCR has been shown to translocate to the lipid rafts where it clusters with several molecules involved in T-cell signalling such as Lck and Zap70 (2, 94, 102). Importantly, the pre-TCR-mediated signalling rescues DN thymocytes from apoptosis and induce proliferation (about 6-8 divisions) as well as further differentiation. Thus, any mutation resulting in an impaired signalling of the pre-TCR causes maturational arrest at the DN3 stage (75, 116). When β-selection is successfully completed thymocytes further mature from the DN4 to the DP stage. During this transition, recombination of the TCRα locus takes place. In parallel, upregulation of CD4 and CD8 occurs that results in the appearance of DP thymocytes that represent about 80-90% of the total thymic cell population.

Thymocytes that express the fully assembled αβTCR undergo further selection steps. Those thymocytes that possess an altered αβTCR complex that is not capable of recognising self-peptide-MHC complexes or to induce proper signals are rapidly eliminated via a process termed "death by neglect" that applies to almost 90% of the thymocytes! Conversely, those thymocytes that possess a functional αβTCR undergo either positive or negative selection. Positive selection enables thymocytes to survive and to complete their developmental program to become T cells, whereas negative selection eliminates autoreactive thymocytes via apoptosis.

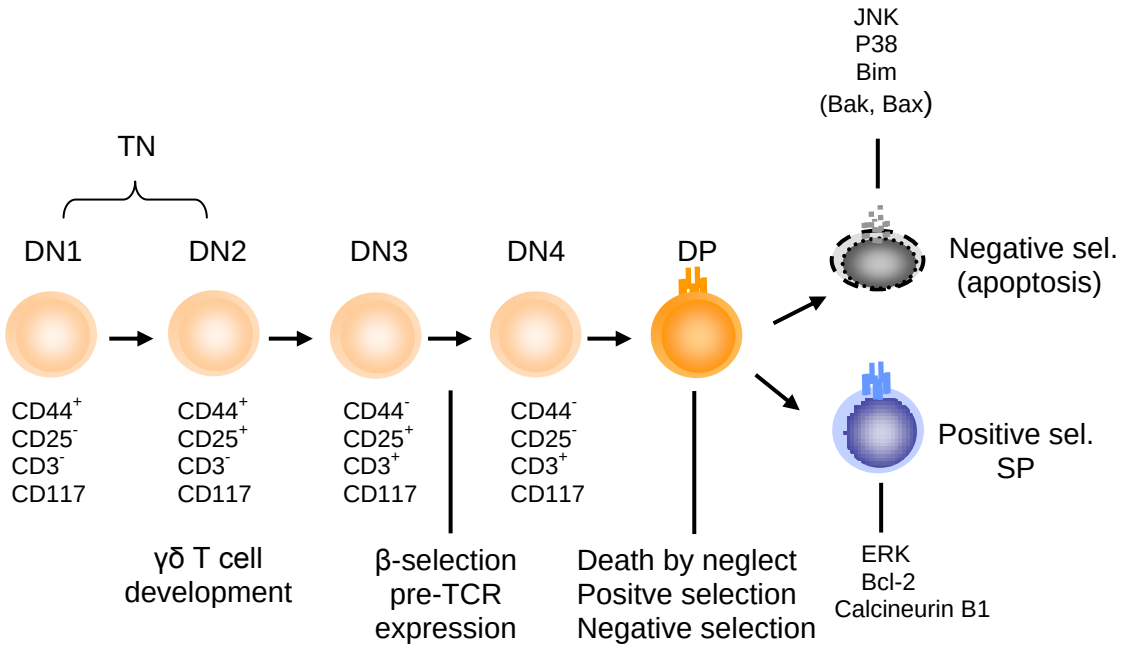


Figure 1. T cell development

Main stages of T-cell development. Major selection events, surface, and intracellular molecules involved in T cell development are indicated. Abbreviations used in this figure: TN (triple negative), DN (double negative), DP (double positive), SP (single positive).

1.2.2. Positive and negative selection of thymocytes

Both positive and negative selection require TCR:self-peptide-MHC contact in the thymus in order to "test" the affinity of the αβTCR complex to self-peptide-MHC. TCRs of immature DP thymocytes undergoing positive selection have low/moderate affinity to self-peptide-MHC complexes. On the contrary, thymocytes expressing a TCR with strong affinity to self-peptide-MHC ultimately die by negative selection. Self-peptide-MHC molecules are expressed on the surface of thymic stromal cells that are in tight contact with developing thymocytes. Thymocyte selection can be monitored by the expression of various surface markers. One protein that is immediately upregulated upon successful engagement with the appropriate self-peptide-MHC is CD69. Expression of CD69 indicates that efficient signalling is generated via the TCR upon engagement with MHC molecules. However, the upregulation of CD69 does not correlate with TCR affinity. Thus, CD69 expression is induced by both positively and negatively selecting ligands and in turn indicates the activation status of thymocytes. Another very useful marker to investigate thymic selection is CD5, a negative regulator of TCR-mediated signalling (7, 103, 114). The expression of CD5 correlates with the signalling strength generated via the TCR. Therefore, analysis of CD5 expression has often been used as an indicator of TCR-mediated signalling strength in several mouse models of thymic development (5-7, 76, 114). Finally, the analysis of the expression of the αβTCR itself is also relevant for the investigation of selection events. Thymocytes that are positively selected such as βTCR^{hi} can be distinguished from βTCR^{lo} cells that are still immature or undergoing negative selection. In conclusion, thymocytes that express high level of CD5, CD69, and TCR will survive and complete their maturation program to become either MHC-I restricted or MHC-II restricted T cells. During thymic selection, the fate of developing T cells largely depends on the affinity of the TCR to self-peptide MHC.

1.2.3. Molecular mechanisms of T-cell development

Although, the general TCR affinity based model of T-cell development is widely accepted (Figure 2) little is known about the molecular mechanisms involved in thymocyte selection. Many efforts have been made to identify molecules that can be exclusively linked to either positive or negative selection processes. However, to date only calcineurin B1 and ERK were reported to be linked exclusively to the positive selection. Calcineurin B1 deficient mice show profound defects in positive selection. However, these cells undergo normal negative selection in various mouse models. Thus, calcineurin B1 seems to be involved in transmission of low affinity signals that

mediate positive selection (76). In addition, the different activation pattern of MAPKs is reported to regulate thymic selection processes. Though it is not entirely clear, but according to a currently proposed model, both the duration and the level of ERK activation is essential to mediate lineage comittement, positive selction but also involved in negative selection (3, 10, 11, 31, 62, 82)**.** Weak but sustained activation of ERK occurs upon low affinity interaction with self-peptide-MHC complexes and results in positive selection of thymocytes, whereas strong and transient ERK activation leads to negative selection (62, 66)**.** In addition both the subcellular localisation and the kinetic of ERK activation were shown to be crutial in terms of determining selection outcome (24). Other MAPKs such as JNK (C-jun NH_2-terminal kinase) have been shown to play a role in negative selection. Transgenic mice expressing a dominant negative form of JNK display an increased resistance to anti-CD3-mediated depletion. This indicates that JNK is involved in deletion of thymocytes undergoing negative selection. This is further supported by the fact that JNK was shown to play a role in mediating apoptosis of various cell types (44, 92).

Thymocytes undergoing negative selection are eliminated by apoptosis. Thus, pro- as well as anti-apoptotic molecules are also involved in regulating T-cell development. Upregulation of the antiapoptotic factor Bcl-2 was shown to be essential during positive selection as it partially rescues thymocytes from apoptosis. (12, 101, 105, 107). In addition, mice lacking the pro-apoptotic factor Bim showed a striking resistance to negative selection in various negative selection models (12). Other important players in mediating apoptosis are Bax and Bak that work in cooperation with Bim to mediate negative selection. Indeed, Bax/Bak double deficient mice show a marked resistance to both death by neglect as well as to antigen induced thymocyte deletion. In conclusion, at the end of thymic selection only those thymocytes survive that recognise self-peptide-MHC complexes with low/moderate affinity (thus are not autoreactive) but capable of fully reacting with foreign antigens (46, 91).

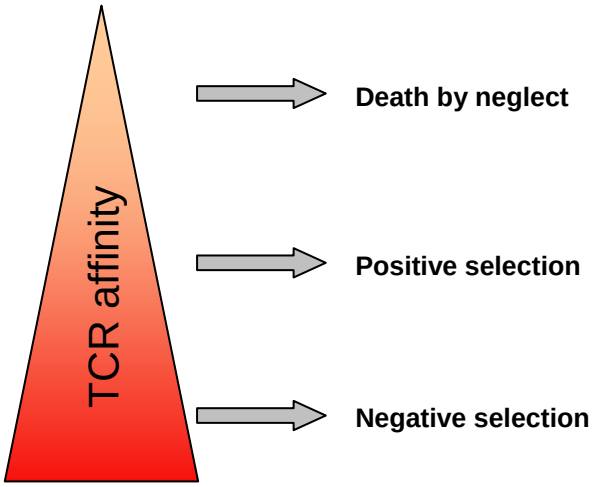


Figure 2. TCR affinity model of T cell development

TCR affinity based model of T-cell development. The increasing colour intensity indicates increasing TCR affinity to self-peptide-MHC complexes. Selection outcomes induced by increasing TCR affinities are shown.

1.3. TCR transgenic mouse models used in this study

1.3.1. MHC-I restricted transgenic mouse models

1.3.1.1. The HY model

HY transgenic mice were generated in 1988 (50). These mice carry a TCR that selectively recognises the male specific antigen derived peptide HY (KCSRNRQYL) in the context of MHC class I H-2D.^{b} In this system, transgenic T cells are committed to the $CD8^{+}$ T-cell lineage (51, 115).

The HY transgenic TCR consists of a TCRβ chain containing the Vβ8 region. The transgenic TCRαβ complex is recognised by the monoclonal antibody clone T3.70. Given the fact that the HY peptide is expressed only in male mice, this system is suitable to investigate both positive and negative thymic selection processes. (6, 26, 51, 103, 115) In fact, in female mice where the HY peptide is not expressed, the transgenic TCR binds to self-peptide-MHC with low affinity, thus inducing positive selection. Conversely, in male mice that express the HY peptide negative selection takes place.

1.3.1.2. P14 transgenic mouse model

P14 TCR transgenic mice were generated in 1989 (86). The P14 transgenic TCR is specific for the gp33 derived peptide KAVYNFATC (aa 33-41) of LCMV presented in the context of MHC-I H2-D^b. Accordingly, T cells are committed to the $CD8^+$ lineage. The transgenic TCR is composed of an α chain containing the Vα2 region paired with a β chain that contains the Vβ8.1 region. The P14 transgenic TCR was shown to have higher affinity than the HY TCR. Indeed, P14 transgenic T cells express higher levels of CD5 and show a more efficient positive selection compared to the HY transgenic mice (7).

1.3.1.3. OT-I transgenic mouse model

OT-I TCR transgenic mice were first generated in 1994 (39). The OT-I transgenic TCR is specific for peptide SIINFEKL. This peptide derives from chicken ovalbumin and is presented in the context of MHC-I H2-K^b. Accordingly, transgenic $CD8^+$ T cells are positively selected on the H-2^b background. The OT-I transgenic TCR consists of TCR chains containing Vα2 and Vβ5 regions. As judged by CD5 expression, the OT-I TCR has a higher affinity than P14 and HY TCRs (6, 48).

1.3.2. MHC-II restricted transgenic mouse models

1.3.2.1. OT-II transgenic mouse model

OT-II transgenic mice were described in 1998 (8). The transgenic TCR expressed in these mice is specific for the chicken ovalbumin peptide epitop aa 323-329 (ISQAVHAAHAEINEAGR) that is presented in the context of MHC I-A^b. Thus, the selection process in OT-II transgenic mice generates $CD4^+$ T cells. The OT-II transgenic TCR contains the Vα2 and the Vβ5 regions. The OT-II transgenic TCR mediates an efficient positive selection of $CD4^+$ T cells.

1.4. Main T-cell populations

1.4.1. αβ and γδ T cells

T lymphocytes can be divided into two distinct groups. The first expresses the γδTCR and the second possesses the conventional αβTCR. T cells expressing the γδTCR represent a distinct population of T lymphocytes. Both in human and mouse, maturation of γδ T cells bifurcates from conventional αβ T cells at the DN2-DN3 stage where the recombination of the γδ loci starts. (9, 57). How $\gamma\delta$ T cells recognise antigens is not entirely clear. However, compelling evidences suggest that it is fundamentally different form αβ T cells. In fact, the antigen recognition process of the

γδTCR is independent from classical MHC molecules and does not require antigen processing. Moreover, it has been shown that γδ T cells recognise lipid antigens presented by the nonclassical MHC-I molecule CD1 (23, 33). Conversely, T cells expressing the αβTCR are restricted either to MHC-II or to MHC-I molecules and possess either the CD4 or the CD8 coreceptors, respectively. Within αβ T cells, $CD8^+$ cells are mainly cytotoxic, whereas $CD4^+$ lymphocytes function as helper or regulatory cells. $CD4^+$ T cells control various aspects of the immune function such as the activity of $CD8^+$ T cells, antibody production, and also antigen presentation by APCs. Thus, $CD4^+$ T cells are indispensable for the organisation as well as for the coordination of the immune response (1, 46).

1.5. T-cell signalling

1.5.1. Structure of the αβTCR complex

The αβTCR is expressed on the surface of all αβ T cells. The TCRβ and the TCRα heterodimer define the antigen-binding groove of the TCR. However, the TCR α and β chains are unable to generate signals. This function is performed by the TCR-associated CD3 chains. The CD3 complex consists of five invariant polypeptide chains γ, δ, ε, ζ, and ή, all of which possess signal transduction abilities. These chains associate and might form the following heterodimers: (ζ ζ), (ζή), (δε), (γε). The (ζζ) form is favoured since about 90% of the surface TCR complexes have (ζζ) whereas only 10% of TCRs contain the (ζή) heterodimer. The link between the different TCR and CD3 subunits is mainly based on ionic bridge interactions. In fact, the αβTCR heterodimer possesses positively charged amino acid residues in the transmembrane region that interact with the negative charged amino acid residues of ζ subunits as well as with other CD3 components. Upon TCR engagement with its cognate ligand, numerous intracellular pathways are activated that at the end culminate in a functional outcome such as proliferation and differentiation. Proximal TCR signalling events are mediated exclusively via ITAMs (immunoreceptor tyrosine-based activation motif) that are localised within the CD3 chains. CD3 γ, δ, and ε possess one ITAM whereas both ζ and ή chains have three ITAMs. Thus, the whole TCR/CD3 complex contains in total 10 ITAMs. The molecular distribution of these motifs is represented in Figure 3.

1.5.2. Proximal signalling events in T cells

A typical ITAM structure has the following aa sequence: YxxL/I/V x_{6-8} YxxL/I/V where x stands for any amino acid. The initiation steps of TCR signalling are dependent on the activity of the Src-family kinases. Upon TCR engagement with its ligand, the members of the Src-family kinases Lck and Fyn phosphorylate tyrosine residues within ITAMs. Thus, phosphorylated ITAMs are essential in mediating signalling events generated via the TCR (42, 46). Once the ITAMs become phosphorylated, they serve as docking sites for Zap70 (zeta associated protein of 70 kDa) a Syk kinase family member. Zap70 is recruited to the phosphorylated ITAMs of CD3 ζζ homodimer via its tandem SH2 domains. Upon binding to ITAMs, Zap70 localises to the proximity of Src kinases such as Lck and Fyn and in turn becomes phosphorylated and thereby activated. The most well-known downstream target of Zap70 is LAT (linker for activation of T cells). LAT plays a crucial role in T-cell development and activation. The cloning and characterisation of LAT was reported in 1988 (125-128). Once phosphorylated on tyrosine residues, LAT recruits cytoplasmic molecules such as Grb2, Gads, PLC-γ. In addition, LAT indirectly associates with several other proteins such as SLP76, Itk, Vav, Rho/Rac and Sos. All these molecules are involved in the formation of a critical signalling complex that links the TCR to more downstream signalling events such as MAPK activation or Ca^{2+} flux (summarized in Figure 3) (56, 125-128).

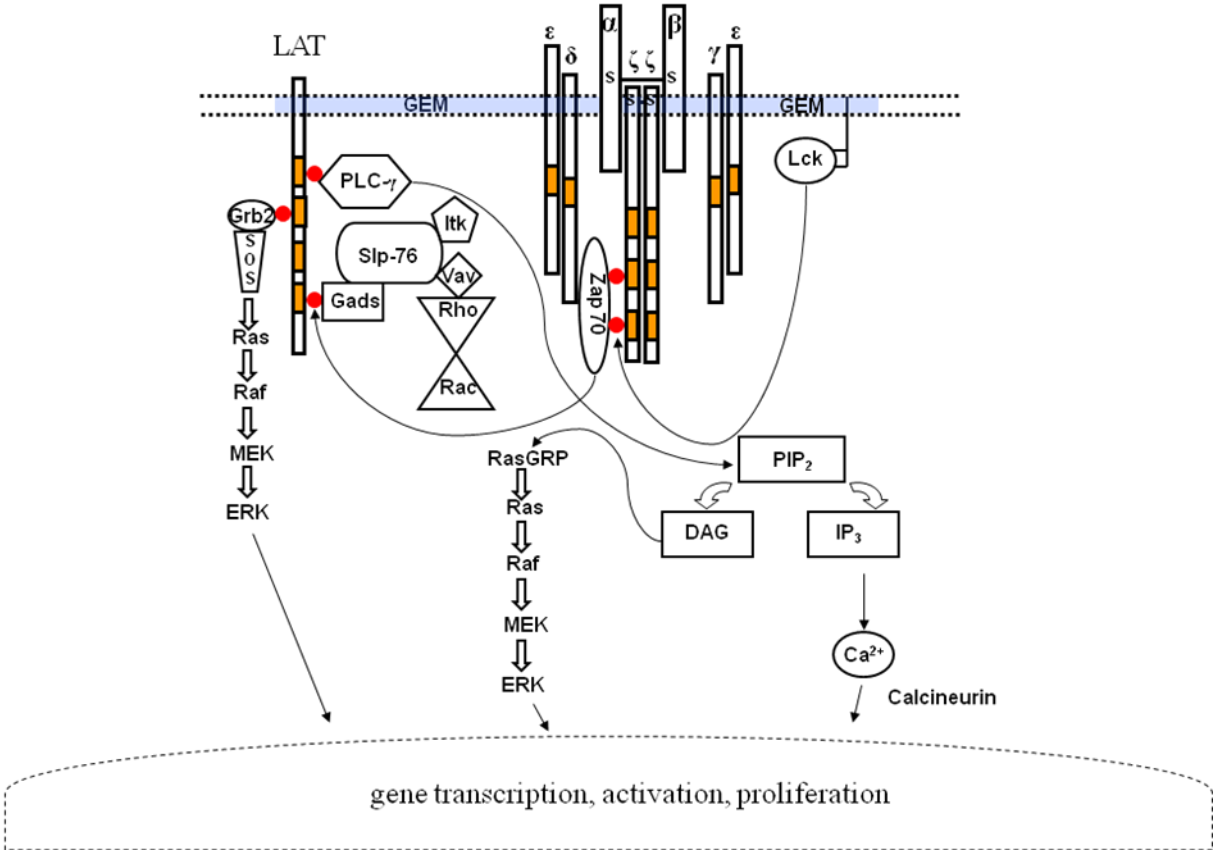


Figure 3. Proximal TCR-mediated signalling events

A typical structure of TCR complex is depicted according to the most frequent arrangement of the CD3 chains. Disulfide bindings between the αβTCR, and ζζ dimers are depicted with black lines. ITAMs are represented as orange boxes. Phosphorylated ITAMs are indicated with red spots. Molecules involved in proximal signalling events are shown. Empty arrows represent signalling pathways whereas black arrows indicate activation.

1.5.3. Transmembrane adaptor proteins (TRAPs)

The discovery of TRAPs was a major achievement in the history of T-cell signalling. All TRAPs possess a short extracellular domain and a long cytoplasmic part. Per definition, TRAPs possess neither enzymatic nor transcriptional activity. All TRAPs contain multiple tyrosine-based signalling motifs (TBSMs) (Figure 4). Upon phosphorylation, TRAPs recruit signalling molecules via TBSMs thereby they organize multiprotein signalling complexes at the plasma membrane. Thus, TRAPs are essential to link membrane receptors to more downstream signalling events. To date, the following TRAPs have been identified: LAT, PAG, NTAL, LIME, that are localised to the GEM fractions and LAX, TRIM, and SIT which are positioned outside of GEMs.

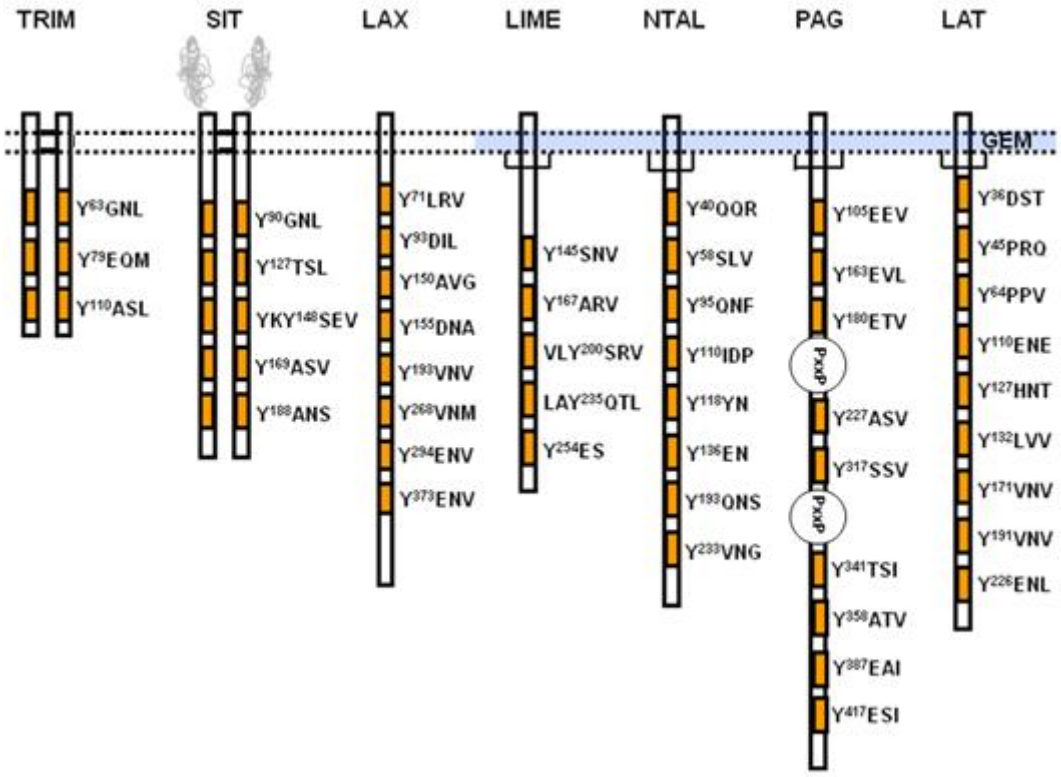


Figure 4. Transmembrane adaptor proteins (TRAPs)

Overall structure of the known TRAPs. The plasma membrane is depicted with either empty or blue boxes indicating non-GEM or GEM membrane compartments, respectively. Tyrosine-based signalling motifs within each adaptor protein are depicted as orange boxes. Other motifs such as proline rich motifs are indicated by circles. The amino acid sequences of TBSMs are shown adjacent to the corresponding motifs.

1.5.4. The SH2-domain-containing protein tyrosine phosphatase (SHP2)-interacting transmembrane adaptor protein (SIT)

1.5.4.1. Structure of SIT

The transmembrane adaptor protein SIT was identified in 1999 (63, 85). SIT is a 30-40kDa disulfide-linked homodimeric polypeptide with a very short extracellular domain (18aa) that contains a single N-glucosylation motif (N^{26}) and one cysteine residue C^{27}, which is involved in the formation of an interchain disulfide bound. The extracellular domain of the protein is followed by a 19aa long intramembrane segment (aa 41-60) as well as by a long cytoplasmic domain (aa 61-196). Although the overall structure of SIT is similar to those of other TRAPs, SIT has a unique feature. In fact, it is the only TRAP possessing an extracellular glycosylation accounting for 20 kDa of its molecular weight. The intracellular domain of SIT contains five TBSMs among which one motif is a conventional ITIM. They are as follows: Y^{90}GNL, Y^{128}TSL, VKY^{148}SEV, Y^{168}ASV, Y^{188}ANS. These motifs represent potential sites for tyrosine phosphorylation. Upon TCR triggering in both HPB-ALL and Jurkat T cells, SIT becomes phosphorylated. The overall level of tyrosine phosphorylations among TBSMs is summarised as follows: YGNL≥YASV>YTSL>ITIM>YANS (63, 85). In addition, both Northern and Western blot analysis revealed that SIT is mainly expressed in lymphoid organs such as the thymus, lymph node and spleen (63, 85, 103).

1.5.4.2. Function of SIT

In Jurkat T cells, SIT was shown to negatively regulate TCR-mediated NF-AT activation. Although it has been shown that, upon TCR engagement, SIT recruits SHP2 via its ITIM, the negative regulatory effect of SIT on TCR-mediated signalling appears to be exclusively mediated via the YASV motif. Additionally, it also has been shown that upon TCR triggering Grb2 binds to SIT via the YGNL-and the YANS-motifs. However, the YGNL motif represents the major Grb2 binding site. The ability of SIT to bind molecules that are important in both positive and negative regulation of TCR-mediated signalling raises the possibility that SIT might have a dual function. Indeed, in the absence of the YASV motif SIT lost its negative regulatory effect on NF-AT activation and instead showed a positive regulatory function mediated via the YGNL motif. Further characterisation revealed that in pervanadate stimulated Jurkat T cells SIT interacts with Csk, a potent negative regulator of the Src family kinases, via the YASV motif. Thus, it is most likely Csk the main candidate to mediate the negative regulatory effect of SIT in Jurkat T cells. In summary, upon TCR stimulation SIT becomes phosphorylated and subsequently recruits SHP2 via its ITIM, Grb2

mainly via its YGNL and Csk via the YASV motif (63, 85). However, the functional relevance of these interactions especially in primary T cells is not yet clear.

1.6. Negative regulation of TCR signalling

1.6.1. Immunoreceptor tyrosine-based inhibitory motifs

The consensus sequence of a typical immunoreceptor tyrosine-based inhibitory motif (ITIM) is S/I/V/LxYxxI/V/L where x stands for any amino acid. ITIMs play an important role in the negative regulation of TCR-mediated signalling by recruiting inhibitory molecules possessing SH2 domains. These molecules are tyrosine and lipid phosphatases such as SHP-1, SHP-2, and SHIP respectively.

1.6.2. SHP-2 (SH2 domain containing protein tyrosine phosphatase) as negative regulator of immunoreceptor signalling

The tyrosine phosphatase SHP-2 is expressed ubiquitously (74). The inhibitory role of SHP-2 in T cells is not entirely clear. The recruitment of SHP-2 to inhibitory receptors is mediated via its tandem SH2 domains that bind to conventional ITIMs. Primary studies have shown that SHP-2 acts as a positive regulator of Ras/ERK pathway downstream of growth factor receptors. In this regard, SHP-2 might counterbalance the function of Csk. However, accumulating evidences suggest that SHP-2 might exert a negative regulatory role in T cells. Data obtained from mice in which the SHP-2 binding site of the common gamma chain receptor (gp130) of IL-6 was mutated suggest a potent negative regulatory role of SHP-2 on cytokine mediated signalling. It seems that SHP-2 dephoshorylates STAT proteins. These mice also developed autoimmunity as well as spleenomegaly further indicating the negative regulatory role of SHP-2 (55, 79, 95). In addition, macrophages that lack SHP-2 display an elevated FcγR-and TLR-mediated IFN production (95). Finally, SHP-2 was shown to associate with CTLA-4 (55). Authors have suggested the presence of an inhibitory complex consisting of SHP-2, CTLA-4, and TCRζ that results in dephosphorylation of the TCRζ chain either directly or indirectly by dephosphorylating Lck, thus resulting in dampening of proximal signalling events (55). Compelling evidences suggest that SHP2 might also inhibit TCR-mediated signalling via the docking protein Gab2 (122, 123). Moreover, the recently described inhibitory molecule BTLA (B and T lymphocyte attenuator) that is upregulated upon T cell activation was shown to recruit SHP-1 and SHP-2. BTLA deficient mice are sensitive to the induction of autoimmune disease. Moreover, T cells from these mice are hyperreactive to CD3 mediated stimuli *in vitro*, further indicating the potential negative regulatory role of SHP-2 in immunoreceptor signalling (120).

1.6.3. The role of Csk as a negative regulator of Src kinases

Csk (C-terminal Src kinase) is a 50 kDa protein tyrosine kinase that is ubiquitously expressed (19, 54, 70). However, its expression is more pronounced in hematopoetic cells. The overall structure of Csk resembles that of Src kinases. It possesses a C-terminal SH3 domain, an SH2 domain, as well as a catalytic domain. Csk is able to inhibit Src kinases by phosphorylating the C-terminal inhibitory tyrosine residue (70). Unfortunately, a more detailed analysis of Csk function is not possible since mice deficient for Csk die at embryonic day 9-10 (43, 71). To circumvent this problem Csk conditional knockout mice were generated. An interesting finding was that immature thymocytes in these mice were able to mature from the DP to the SP $CD4^+$ stage. Of note, development of $CD4^+$ T cells in the absence of Csk was MHC-II independent (97). Thus, constitutive activation of Src kinases abrogates the need for proper pre-TCR signalling and MHC restriction in developing CD4 T cells. Moreover, Csk overexpression studies in a T-cell line (BI-141) revealed a profound inhibition of global tyrosine phosphorylation and IL-2 production upon TCR stimulation. These findings indicate that Csk-mediated regulation of Src kinases is essential during T-cell development and peripheral T-cell function (18). Csk constitutively associates with the PEST domain-enriched tyrosine phosphatase (PEP) that is expressed only in haematopoietic lineages (21). In T-cell lines, PEP overexpression was shown to inhibit TCR mediated signalling. (22) Thus, Csk might also exert its negative regulatory function via PEP. Taken together, Csk negatively regulates the activation status of Src kinases and thus, has a strong impact on both T-cell development and function.

1.6.4. CD5 is a negative regulator of TCR-mediated signalling

The CD5 molecule belongs to the scavenger receptor cysteine rich B (SRCR) family. The extracellular part of CD5 contains three SRCR domains followed by a hydrophobic transmembrane region and a relatively long intracellular domain. The human CD5 molecule was cloned in 1986 whereas the mouse CD5 was cloned in 1987. The intracellular part of CD5 contains four tyrosine residues among which two are functionally important. Tyr441 is located within an imperfect ITAM motif whereas Tyr378 is a part of an ITIM-like motif. CD5 was shown to become tyrosine phosphorylated upon TCR triggering most likely by Lck (88). Once phosphorylated, CD5 recruits PI3K (p85) via its ITAM-like motif and SHP-1 via its ITIM-like motif (15, 25, 27, 88). Additionally, it has been shown that CD5 colocalises with the TCR

complex (81). Thymocytes from $CD5^{-/-}$ mice are hyperreactive and show an enhanced proliferation and Ca^{2+} mobilisation upon TCR triggering *in vitro*. The lowered threshold of activation leads to an altered T-cell development in these mice. When crossed onto HY or P14 transgenic background $CD5^{-/-}$ mice showed a more activated phenotype as well as an enhanced positive selection (6, 114). In addition, signalling molecules that are essential for proper T-cell activation such as LAT and Vav were shown to be hyperphosphorylated in CD5 deficient thymocytes. Taken together, these data suggest that CD5 acts as a potent negative regulator of TCR mediated signalling by recruiting SHP-1 to the plasma membrane via its ITIM-like motif (83). Thus, CD5 might contribute in a negative feedback loop that counterbalances TCR-mediated signalling. This view is further supported by the fact that the surface expression of CD5 on T cells is proportional to the affinity of the TCR (6, 7).

1.7. T-cell homeostasis

1.7.1. The role of different factors in T-cell homeostasis

The number of peripheral T cells is tightly regulated during the course of life. The mechanism that is responsible for maintaining peripheral T-cell numbers relatively constant is termed as T-cell homeostasis. Various mechanisms contribute to regulate the size of the peripheral T-cell pool. In young mice, the thymus plays a major role in T-cell pool maintenance by generating naïve T cells. However, survival and self-renewal of naïve T cells contribute to maintain constant T-cell numbers during adulthood. Peripheral T cells require continuous signals to survive. These survival signals are both TCR and cytokine mediated. T cells deprived of such signals die rapidly. An experimental model to investigate T-cell homeostasis (survival and self renewal capacity) is the adoptive transfer of small numbers of T cells into lymphopenic recipients. Under lymphopenic conditions, adoptively transferred T cells undergo rapid proliferation to repopulate the “empty niche”. Natural lymphopenia driven T-cell proliferation occurs after chemotherapy, or in AIDS patients, as well as patients suffering from other diseases causing severe immunodeficiency (29, 36, 61). In addition, a partial lymphopenic situation occurs during early ontogenesis. For instance, newborn mice possess only few T cells in the periphery. Thus, they are considered to be partially lymphopenic.

Experimental mouse models of lymphopenia are sublethaly irradiatiated mice or *rag1/rag2 deficient* mice. An additional way to induce lymphopenia is injecting T-cell depleting antibodies (78). The capability of adoptively transferred T cells to expand in

a lymphopenic host is a measure of the homeostatic „fitness" or self-renewal capacity of the donor cells (47).

1.7.2. Homeostatic proliferation of T cells

The factors that mediate T-cell survival and homeostasis are not entirely clear. Accumulating evidences suggest that both $CD4^+$ and $CD8^+$ naïve T cells require continuous low affinity TCR:self-peptide-MHC induced signals to survive upon adoptive transfer (13, 30, 35, 47, 64, 69, 77, 100, 111, 113). In line with these observations low affinity peptides that mediate positive selection in the thymus have been shown to be involved in regulating peripheral T-cell survival and homeostasis of both MHC-I and MHC-II restricted T cells (30, 35). Studies performed on TCR transgenic mouse models have revealed that "homeostatic fitness" of T cells correlates with the avidity of the TCR (47, 48). T cells that express TCR with higher avidity have proliferative advantage over the cells possessing TCR with lower avidity when adoptively transferred into lymphopenic hosts (30, 48, 108). Indeed, adoptively transferred T cells from TCR transgenic mice carrying TCRs with different affinities display different capability to proliferate under lymphopenic conditions (13, 30, 35, 47, 64, 69, 77, 93, 111, 113) . In line with these observation transgenic $CD8^+$ T cells carrying the low affinity HY TCR are unable to undergo homeostatic proliferation in lymphopenic hosts (30, 77, 113). The fact that T-cell proliferation and survival capacity in lymphopenic hosts are proportional with TCR affinity, indicates that the strength of TCR-mediated signal is crucial in setting the homeostatic capability of T cells.

1.7.3. The role of cytokines in T-cell survival and homeostasis

In addition to proper TCR:self-peptide-MHC interaction cytokines, especially IL-7 and IL-15 are also essential in mediating T-cell survival and homeostasis. Studies utilizing IL-7Rα deficient mice have shown that signalling via IL7Rα is essential for homeostatic proliferation of naïve T cells in lymphopenic hosts (38, 98, 99). In line with this finding, knockout mice lacking either IL-7Rα or IL-7 display a severe lymphoid hypoplasia, thus demonstrating the potent role of IL-7 in T-cell homeostasis and survival. Treatment of thymectomized mice with IL-7 blocking antibodies leads to a reduction of Bcl-2 levels in T cells thus, results in shortening the half-life of peripheral T cells (58, 90, 96, 118). However, the contribution of cytokines to T cell homeostasis might also depend on the degree of lymphopenia. Under extreme lymphopenic conditions IL-7R mediated signals seem to play a primary role whereas under less severe lymphopenia TCR:self-peptide-MHC mediated signals are

essential to induce lymphopenia induced expansion of T cells (99). In contrast to naïve T cells, survival and homeostasis of memory T cells is less dependent on TCR: self-peptide-MHC interaction but rather requires cytokines (47, 69, 109). The main cytokine that triggers survival and proliferation of memory $CD8^+$ T cells is IL-15 (58, 129). $CD8^+$ memory T cells are able to survive if IL-15 is present even in the absence of TCR-derived signals (69). On the contrary, IL-15 seems to be dispensable in memory $CD4^+$ T-cell survival and homeostasis indicating that homeostasis of memory CD4 and CD8 T cells is likely differentially regulated (112). In conclusion, survival and homeostasis of both $CD4^+$ and $CD8^+$ T cells require TCR:self-peptide-MHC mediated signals yet, the relative contribution to the homeostatic response by TCR and cytokine signals critically depends on both the degree of lymphopenia and phenotypic characteristics of individual T cells.

1.7.4. Phenotype of T cells undergoing lymphopenia-induced expansion

T cells undergoing homeostatic expansion stepwise acquire a memory-like phenotype as they upregulate some memory markers such as CD44, CD122 (IL-2/IL-15Rβ) (17, 32, 37, 45, 68). However, they keep the expression of the naive T-cell marker, CD62L. Moreover, T cells undergoing lymphopenia-induced proliferation do not become fully activated and do not upregulate activation markers such as CD69 or CD25. Finally, functional assays revealed that TCR-mediated response (IFNγ, IL-2 production, proliferation) of these cells partially resembles those of memory cells though with less amplitude. Thus, T cells undergoing homeostatic proliferation show a bimodal profile that resembles to that of both memory and naïve T cells (17, 45, 68, 80).

2. Results

2.1. Genotyping SIT deficient mice

SIT-deficient mice were generated in close cooperation with Dr. Klaus Pfeffer using a *sit* gene targeting construct designed by Dr. Eddy Bryns (Figure 5A). Briefly, the *sit* gene was disrupted by a standard gene targeting strategy. The entire coding sequence of SIT was replaced by a neomycin resistance cassette. Targeted mutation and Mendelian segregation was further assessed by genotyping SIT-deficient mice by standard PCR using genomic DNA purified from mouse-tail tissue. Figure 5B shows PCR products typically obtained from wild-type, heterozygote, and SIT knockout mice. The PCR product of the wild-type allele is 300 base pair long while the product of the muated allele is 500 base pair long. As expected, PCR products obtained from heterozygote mice show two distinct bands corresponding to the PCR products obtained from both wild-type and mutant alleles, respectively.

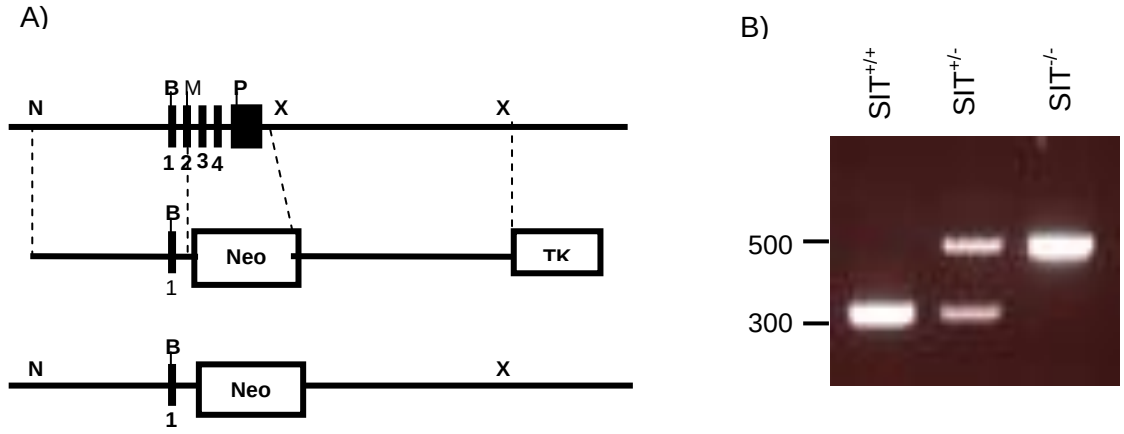


Figure 5. SIT gene targeting and genotyping strategies

A) Partial restriction map of the SIT locus and the targeting cassette. Filled boxes indicate exons. Restriction sites are indicated as follows: N (NotI), B (BamHI), M (MluI) P (PstI), X (XbaI). Abbreviations: NEO: neomycin, TK: thymidine kinase. B) Genomic DNA was purified from wild-type, heterozygote or SIT-deficient mice from tail tissue. Subsequently a standard PCR reaction was carried out. PCR products were typically loaded onto 2% agarose gel. PCR products are shown from $SIT^{+/+}$, $SIT^{+/-}$ and $SIT^{-/-}$ mice, respectively. Corresponding marker lanes are indicated on the left side.

2.2. Expression of SIT in mice

2.2.1. SIT is expressed predominantly in T cells

Previous analysis of different mouse tissues revealed that SIT is expressed mainly in primary and secondary lymphoid organs (63, 103). In order to further determine the expression of SIT in mouse primary cells, lysates were prepared from lymphoid organs and SIT expression was subsequently determined by standard western blot. In agreement with previous results, SIT is abundantly expressed in the thymus but less in the spleen (Figure 6). Moreover, among primary cells, SIT is strongly expressed in $CD3^+$ T cells but it is hardly detectable in $B220^+$ B cells. Thus, SIT is mainly expressed in both immature and mature T cells.

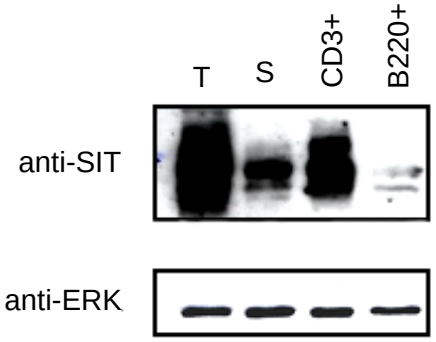


Figure 6. SIT is mainly expressed in T cells

SIT expression in primary mouse cells. Thymocytes (lane 1) total spleen cells (lane 2), purified T cells (lane 3) and purified B cells (lane 4) were isolated from wild-type mice, lysed and subsequently an SDS PAGE was carried out. Membrane was probed with anti-SIT antibody. Equal protein loading is shown by reprobing the membrane with an anti-ERK antibody.

2.3. The role of SIT in T-cell development

2.3.1. $SIT^{-/-}$ mice show normal B-cell, but altered T-cell development

SIT deficient mice were born at the expected Mendelian frequency, they were viable, fertile, and showed no signs of abnormalities. Flow cytometry analysis revealed alteration neither in B-cell development nor in the distribution of B-cell subsets in the bone marrow, spleen or lymph nodes (Figure 7A and (103)). However, FACS analysis revealed that SIT-deficient mice had an altered T-cell development. Figure 7B shows that there is a marked reduction in both percentages and absolute numbers of both $CD8^+$ and $CD4^+$ SP thymocytes (see also (103)). Conversely, I observed an increased proportion of DP thymocytes in $SIT^{-/-}$ mice. In agreement with these observations, a significant decrease of thymocytes expressing high levels of TCR (as a sign of terminal maturation) was found in $SIT^{-/-}$ animals (Figure 7B)**.** Nonetheless, the early stages of thymic development were found to be normal in $SIT^{-/-}$ mice (Figure 7C). These results indicate that SIT is dispensable during the early steps of thymic development but it is required during the transition from the DP to the SP stage.

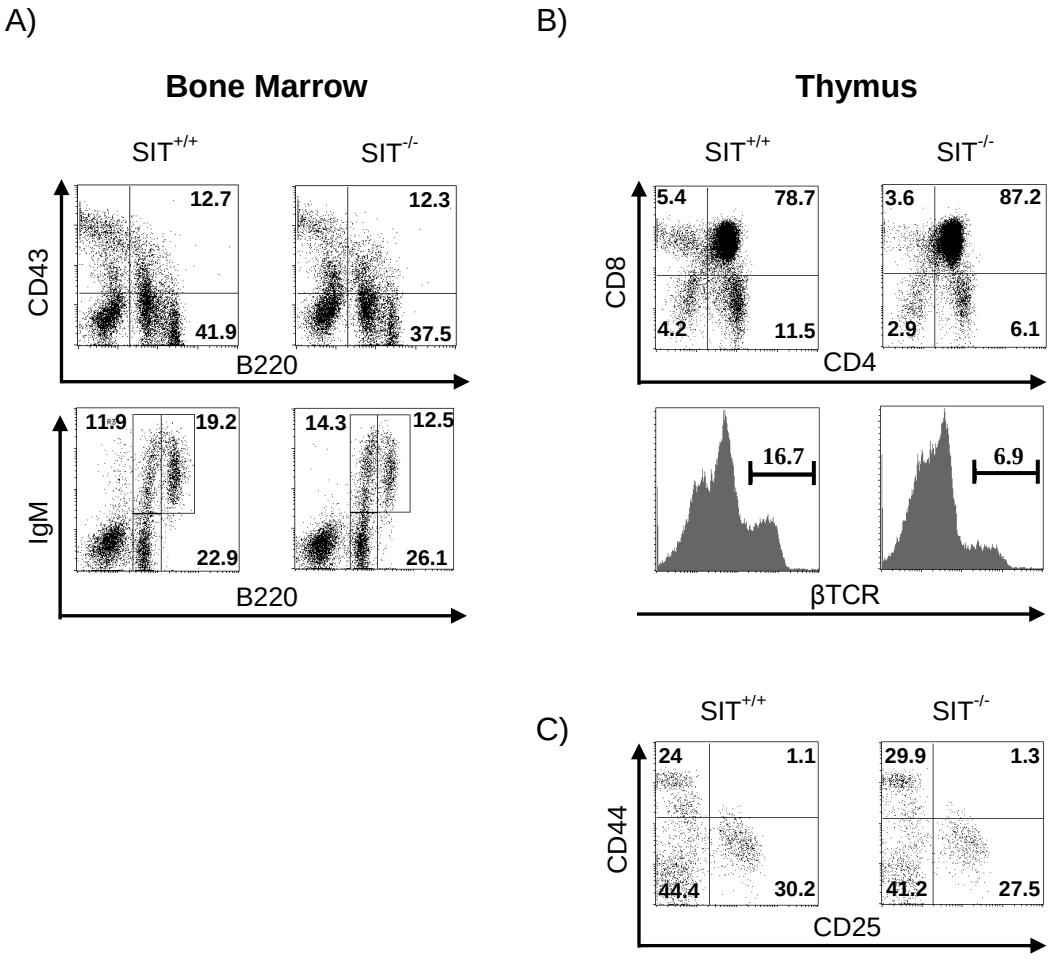


Figure 7. Normal B-cell but altered T-cell development in SIT$^{-/-}$ mice

A) Bone marrow cells were isolated from both wild-type and SIT-deficient mice and subsequently analysed by flow cytometry for the expression of IgM, B220, and CD43. B) Thymocytes from SIT$^{+/+}$ and SIT$^{-/-}$ mice were isolated and single cell suspensions were stained for CD4, CD8, and βTCR. C) Early T-cell development. Thymocytes from wild-type and SIT-knockout mice were isolated and subsequently stained for CD44 and CD25. Numbers indicate percentages of cells within each quadrants or region. All FACS profiles shown here are representative of multiple experiments.

2.3.2. SIT- deficient thymocytes show an activated phenotype

To shed further light onto the alteration of thymic development in SIT$^{-/-}$ mice, I analysed the expression levels of surface markers whose expression is differentially regulated during thymic development. First, I investigated CD5 the expression of which directly correlates with the intensity of pre-TCR and TCR-mediated signalling (6, 7). Flow cytometric analysis revealed that SIT$^{-/-}$ mice display an elevated expression of CD5 on total as well as on DP thymocytes. Next, I studied the expression of the activation marker CD69, which has been shown to be differentially regulated during T-cell development (5). Similar to CD5, CD69 expression was also found to be upregulated in both total and DP thymocytes from SIT$^{-/-}$ mice (Figure 8). These results indicate that SIT$^{-/-}$ thymocytes receive a stronger signal via the TCR and in turn display a more activated phenotype. Thus, loss of SIT results in a lowered threshold of TCR-mediated activation in thymocytes.

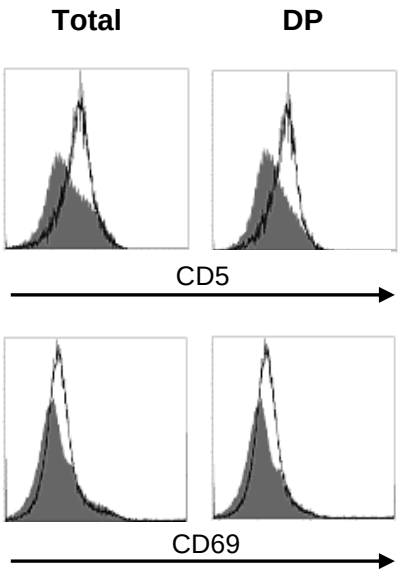


Figure 8. SIT$^{-/-}$ thymocytes show a more activated phenotype

Representative histogram overlays of CD5 and CD69 expression on DP or total thymocytes derived from wild-type (shaded histograms) or SIT-deficient (empty hitograms) mice. Single cell suspensions of thymocytes were prepared and stained with CD4, CD8, CD69 and CD5 antibodies.

2.3.3. Enhanced positive selection in MHC-I restricted transgenic mice

In order to study the role of SIT in positive and negative selection, I took the advantage of TCR transgenic mice. Usage of TCR transgenic mice is reported to be suitable to study thymic selection processes (6, 20, 50). First, I used the HY transgenic mouse system that enabled us to analyse of both positive and negative selection. The HY transgenic TCR is thought to have low affinity to its selecting ligand (28). Thus, we hypothesised that in the absence of SIT the signalling of the HY specific TCR would become stronger. This would result in a more efficient positive selection. To test this hypothesis, I characterised T-cell development of HY TCR transgenic SIT-deficient mice.

Flow cytometric analysis showed that the expression of CD5 was elevated on both DP and positively selected $CD8^+$ SP cells from $SIT^{-/-}$ HY tg female mice (Figure 9A right panel). This observation indicates that indeed thymocytes receive a stronger signal via TCR in these mice. To directly demonstrate that SIT regulates TCR signalling threshold, I investigated the activation of ERK, a downstream effector molecule induced upon TCR triggering. Figure 9A shows that the level of phosphorylated ERK was higher in DP thymocytes from SIT-deficient HY transgenic mice compared to wild-type HY transgenic animals (MFI 59.0 vs. 30.4). Remarkably, the level of phosphorylated ERK was similar after *in vitro* stimulation with PMA (MFI 170 vs. MFI 183), ruling out the possibility that cells express different amounts of phosphorylable ERK or display an overall decreased ability to phosphorylate ERK (Figure 9A and (103)). Thus, as expected SIT deficiency resulted in a stronger signalling via the TCR.

In addition, an interesting observation was that the proportion of non-transgenic $CD4^+$ T cells was strongly reduced in $SIT^{-/-}$ HY transgenic mice. Normally, this population arises after a pairing of the transgenic TCRβ chain with non-transgenic TCRα chains. However, in SIT-deficient HY transgenic mice the enhanced HY TCR-mediated signalling resulted in a more efficient suppression of further rearrangement of the TCRα locus. This resulted in a reduced proportion of non-transgenic $CD4^+$ T cells (Figure 9B lower panel).

Taken together, the elevated expression of CD5, the more mature phenotype, a more activated ERK, and a more effective suppression of the recombination of non-transgenic TCRα locus clearly indicate that loss of SIT resulted in an enhanced positive selection in the HY transgenic mouse model.

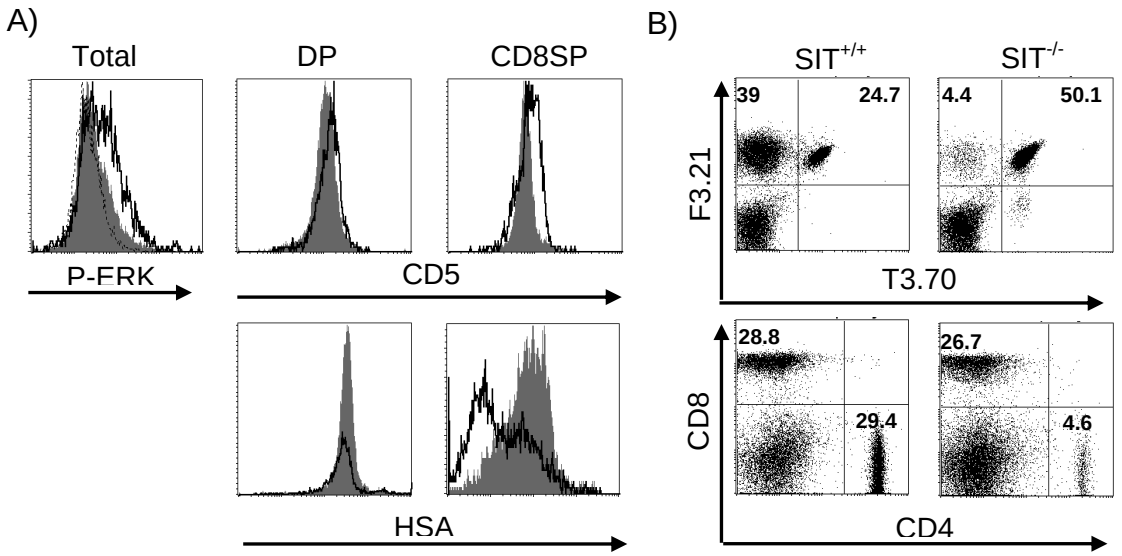


Figure 9. Enhanced positive selection in SIT$^{-/-}$ HY transgenic mice

A) Thymocytes from HY TCR tg SIT$^{+/+}$ (shaded) or from HY TCR tg SIT$^{-/-}$ mice (empty) were isolated, fixed, permeabilized and stained intracellulary to determine the level of phosphorylated ERK. Corresponding staining with isotype control antibody is indicated with a dotted line. Expression levels of CD5 and HSA on gated DP or CD8 SP thymocytes (right upper and lower panels). B) Upper panel: lymph node cells from SIT$^{+/+}$ and SIT$^{-/-}$ HY transgenic mice were isolated and stained with the T3.70 antibody, recognizing the clonotypic transgenic TCRα chain and with F3.21 antibody recognizing the transgenic TCRβ chain, respectively. Lower panel: expression of CD4 and CD8 on lymph node cells of HY transgenic SIT$^{+/+}$ and SIT$^{-/-}$ mice. Numbers indicate percentages in each quadrant.

To further test the consequences of a more efficient TCR-mediated signalling on thymocyte differentiation I also studied the level of HSA (CD24), a marker whose expression inversely correlates with the maturation status of thymocytes. As expected, flow cytometric analysis revealed that SIT$^{-/-}$ HY transgenic thymocytes expressed strikingly less HSA indicating that they displayed a more mature phenotype (Figure 9A lower panel).

2.3.4. SIT deficiency resulted in a conversion from positive to negative selection in TCR transgenic mice

Based on the model (depicted in Figure 2) we expected that loss of SIT would result in a shift from positive to negative selection. Indeed, I observed a reduction in the total number of HY TCR transgenic DP thymocytes in SIT-deficient mice (Figure 10A). Thus, loss of SIT not only resulted in enhanced positive selection but also induced a partial conversion from positive to negative selection.

Similar to HY transgenic mice, SIT deficient P14 mice also showed a marked reduction in the number of DP thymocytes and displayed an even more dramatically reduced thymic cellularity (Figure 10B). Thus, loss of SIT resulted in a shift from positive to negative selection also in P14 transgenic mice.

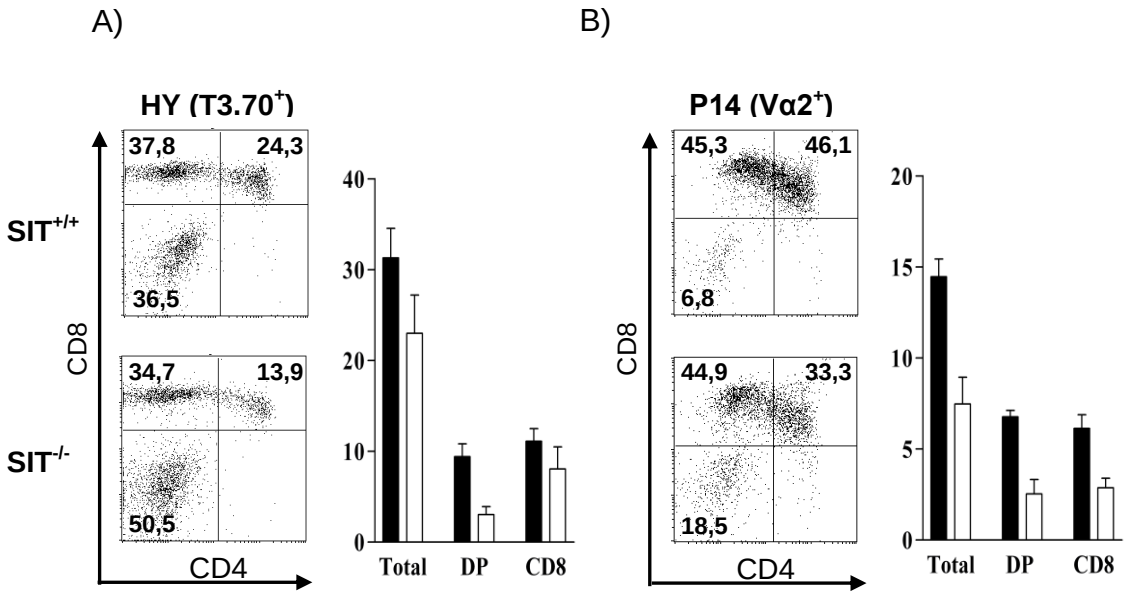


Figure 10. Conversion from positive to negative selection in HY and P14 TCR tg $SIT^{-/-}$ mice

Single cell suspensions were prepared from HY (A) and P14 (B) transgenic $SIT^{+/+}$ and $SIT^{-/-}$ mice and subsequently stained for CD4, CD8, and the transgenic TCR expression. Expression profile of CD4 and CD8 on gated transgenic thymocytes is shown. Numbers indicate percentages in each quadrant. Bar graphs show cellularity ($x10^6$) of each thymocyte subset. Black bars represent wild-type, whereas empty bars indicate SIT-deficient transgenic mice.

Finally, I studied the effect of the loss of SIT on the development of OT-I TCR transgenic T cells, which express a TCR with strong affinity. Surprisingly, I found no alteration of thymic development in $SIT^{-/-}$OT-I transgenic mice (Figure 11 right panel). In addition, I also wished to investigate whether SIT has an effect on the development of $CD4^+$ T cells. To this end, I investigated thymic development in MHC-II restricted OT-II transgenic mice. However, I did not find any alteration of T-cell development in these mice (Figure 11 left panel). Therefore, we hypothesised that SIT influences the development of $CD8^+$ T cells that carry TCR with low or intermediate affinity (see also Figure 13).

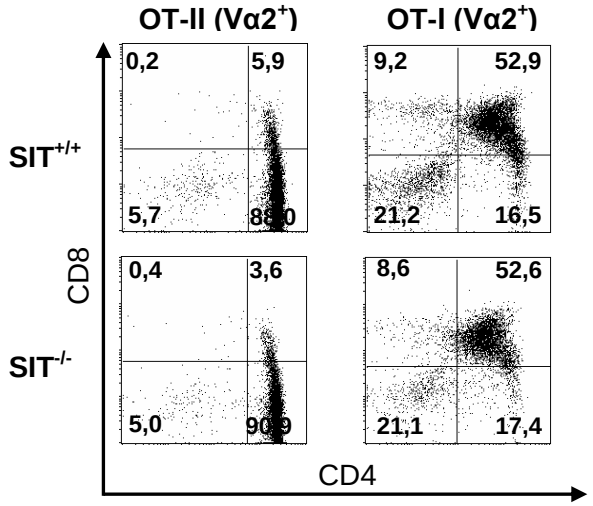


Figure 11. SIT is dispensable for the development of OT-I and OT-II transgenic T cells

Single cell suspensions of thymocytes from $SIT^{+/+}$ and $SIT^{-/-}$ OT-II and OT-I transgenic mice were prepared and stained for CD4, CD8, and transgenic (Vα2) TCR expression. Numbers indicate percentages of cells in each quadrant.

2.3.5. Normal negative selection in the absence of SIT

In order to find out whether SIT plays a role in negative selection, I also investigated SIT-deficient and wild-type TCR transgenic HY male mice. As indicated in figure 12, I could not find any obvious differences between thymocytes from $SIT^{+/+}$ and $SIT^{-/-}$ HY transgenic male mice. This result indicates that loss of SIT does not have a direct effect on negative selection at least in the HY transgenic mouse model (103).

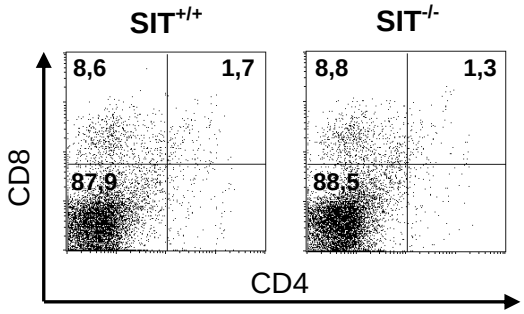


Figure 12. Normal negative selection in HY male TCR transgenic $SIT^{-/-}$ mice

Thymocytes were isolated from either wild-type or SIT-deficient male HY transgenic mice. Subsequently cells were stained with CD4 ,CD8, and T3.70 antibodies and analysed by flow cytometry. Representative profiles showing CD4 and CD8 expression of gated transgenic $T3.70^{+}$ thymocytes are shown.

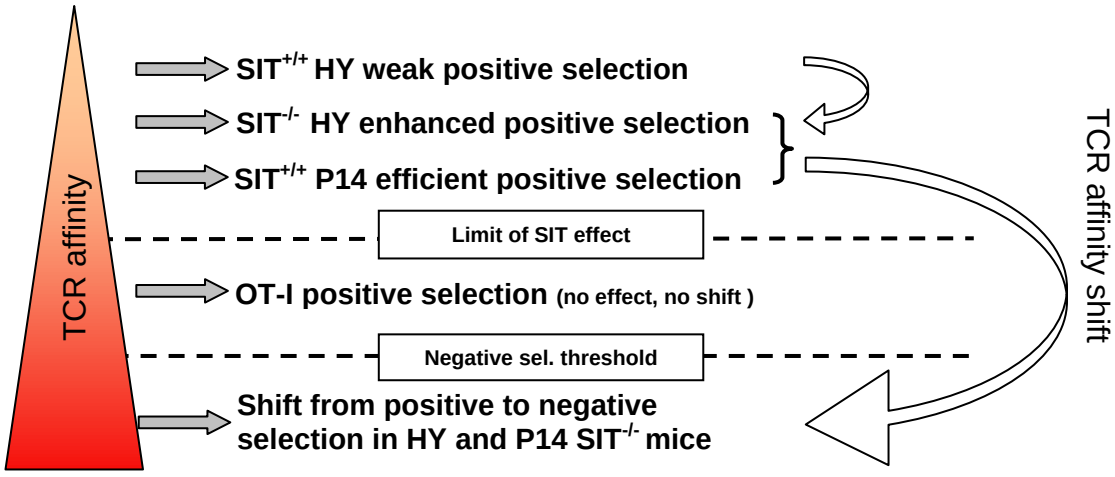


Figure 13. Effect of SIT on T-cell development

Increasing colour intensity indicates increasing TCR affinities. Selection outcomes of MHC-I restricted SIT-deficient and wild-type TCR transgenic thymocytes are indicated. Empty arrows represent the shift of TCR affinity caused by the loss of SIT.

2.4. Altered distribution of peripheral T-cell subsets in SIT$^{-/-}$ mice

2.4.1. Reduced peripheral T-cell numbers in SIT-deficient mice

In order to investigate whether SIT deficiency also resulted in an alteration of the distribution of peripheral T-cell pools, first I investigated the cellularity of peripheral lymphoid organs from SIT-deficient mice. As figure 14 shows, SIT deficiency resulted in a reduction of peripheral CD8$^+$ T-cell number in both spleen and lymph nodes, whereas CD4$^+$ T cells were only slightly affected. (see also (103)).

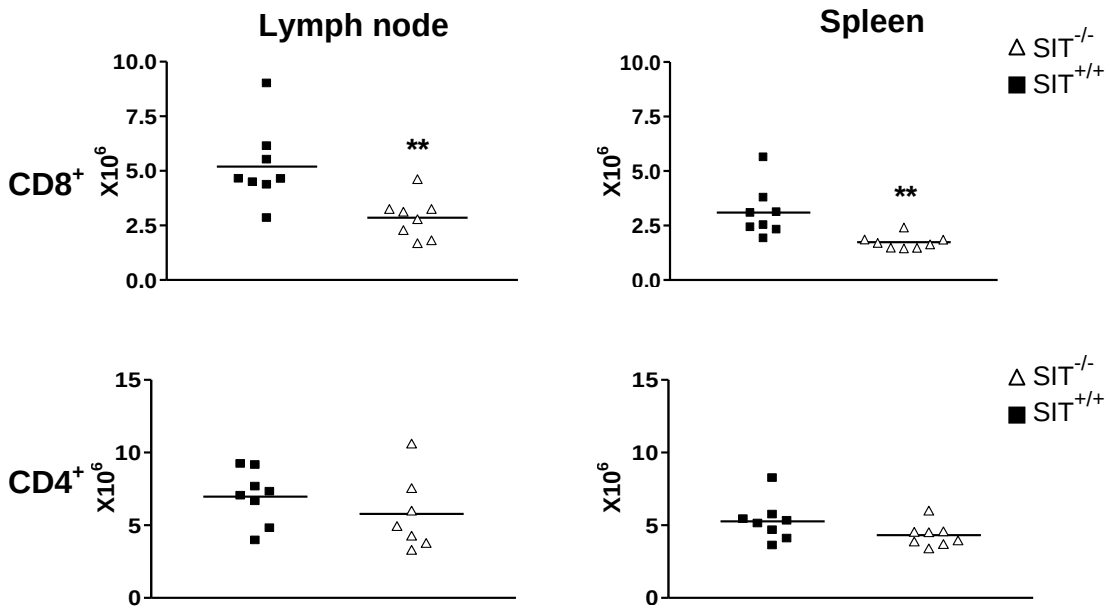


Figure 14. Altered T-cell subsets in SIT-deficient mice

Numbers of CD4$^+$ and CD8$^+$ T cells in lymph nodes and spleen from wild-type and SIT-deficient mice. Lymph node and spleen cell suspensions were prepared form 3 weeks old wild-type and SIT-deficient mice and stained for CD4 and CD8 expression. Based on FACS analysis, absolute numbers of CD4 and CD8 T cells were calculated.

2.4.2. SIT-deficiency resulted in alteration of naïve $CD8^+$ T cells

The peripheral T-cell pool consists of naive and memory subpopulations that express low and high levels of CD44, respectively. To find out which T-cell subset was affected by the loss of SIT, I performed a detailed characterisation of both naïve and memory $CD4^+$ and $CD8^+$ cells. Figure 15 indicates that the number of $CD44^{low}CD8^+$ naïve T cells was strongly reduced both in the spleen and lymph nodes of SIT-deficient mice (Figure 15, left panel). On the contrary, naïve $CD4^+$ T cells were not significantly altered by the loss of SIT (Figure 15 right panel).

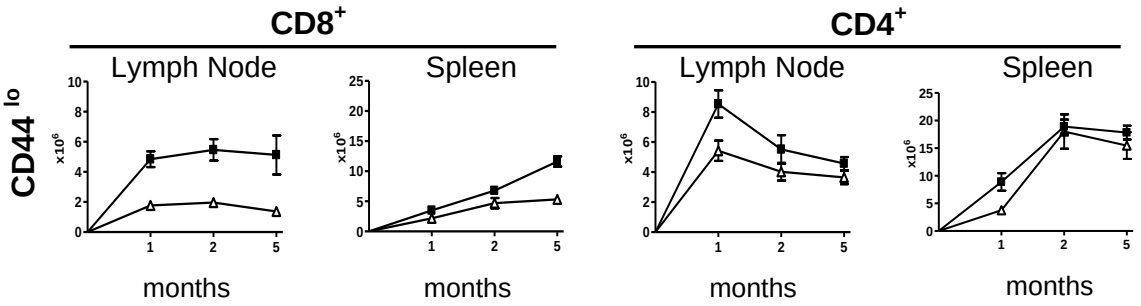


Figure 15. Decreased number of naïve $CD8^+CD44^{low}$ cells in $SIT^{-/-}$ mice

Absolute numbers of $CD8^+CD44^{low}$ and $CD4^+CD44^{low}$ T cells in lymph node and spleen. Based on CD44 expression absolute numbers of $CD4^+$ and $CD8^+$ $CD44^{low}$ T cells were calculated. Mean±Standard Error of the Mean values (SEM) are shown in each graph. Empty triangles indicate SIT-knockout whereas filled squares represent wild-type mice.

As our data suggested that, the loss of SIT more strongly affected $CD8^+$ T cells, I further investigated T-cell homeostasis in $SIT^{-/-}$ mice. In young mice, thymic output is essential to keep naive T-cell numbers constant in the periphery. I showed previously that the generation of SP thymocytes was reduced in $SIT^{-/-}$ mice (Figure 7). The reduced generation of T-cell precursors in the thymus appeared to correlate with the reduced number of naïve T cells in SIT-deficient mice. Recent thymic emigrants (RTEs) are defined as T cells with direct thymic origin. They can be identified by the expression of CD103 (αE integrin) (65, 106). Therefore, I analysed whether RTEs are affected in SIT-deficient mice. As expected, $SIT^{-/-}$ mice displayed a marked reduction in the percentage of RTEs ($CD44^{low}CD103^+$) indicating that the loss of naïve $CD8^+$ T cells is at least partially due to the reduced generation of T-cell precursors (Figure 16).

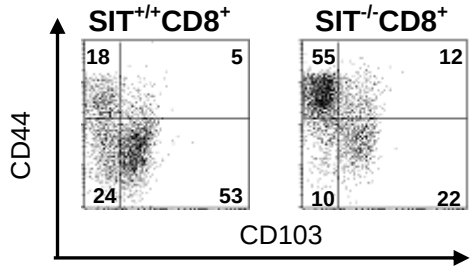


Figure 16. Reduced proportion of recent thymic emigrants in $SIT^{-/-}$ mice

Representative CD44 and CD103 expression profiles of $CD8^+$ gated spleen T cells. Single cell suspensions were prepared from spleen from both wild-type and SIT knockout mice and stained with CD8, CD44 and CD103 antibodies. Numbers indicate percentages in each quadrant.

2.4.3. The decreased number of $CD8^+$ naive T cells in $SIT^{-/-}$ mice is not due to survival defect

To further study whether an intrinsic T-cell survival defect might also contribute to the loss of naive $CD8^+$ T cells in SIT-deficient mice, I tested the survival capacity of lymph node T cells *in vitro.* Total lymph node cells from $SIT^{+/+}$ and $SIT^{-/-}$ mice were prepared and cultured in medium for three days. Percentages of living cells were assessed by FACS analysis. However, survival of SIT deficient and wild-type lymph node cells was comparable (Figure 17A). In agreement with these data, the antiapoptotic factor Bcl-2, whose expression is reported to directly correlate with T-cell survival, was also equally expressed in $SIT^{+/+}$ and $SIT^{-/-}CD8^+$ T cells (Figure 17B) (49, 53). An additional mechanism that might also contribute to the loss of naive $CD8^+$ T cells could be an increased apoptosis. To address this issue, I stained *ex vivo* isolated lymph node cells and spleenocytes with Annexin V. However, I found no differences between SIT-deficient and wild-type $CD8^+$ T cells (Figure 17C). Taken together, these observations suggest that the loss of naïve $CD8^+$ T cells observed in $SIT^{-/-}$ mice is not due to an intrinsic survival defect.

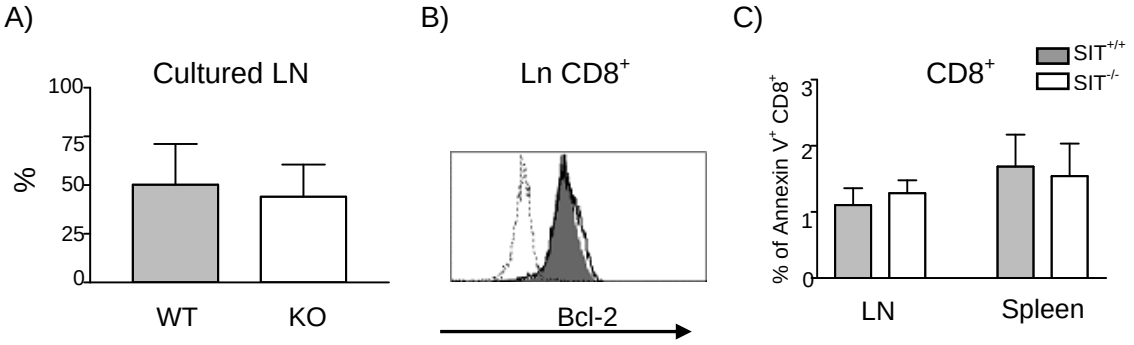


Figure 17. Normal survival rate of $SIT^{-/-}$ $CD8^+$ T cells

A) Lymph node cells (LN) from $SIT^{+/+}$ and $SIT^{-/-}$ mice were prepared and cultured for 3 days in mouse medium and subsequently analysed by FACS. Bar graphs grey (wild-type) and empty (knockout) indicate percentage of gated living cells. B) Expression of Bcl-2 in $SIT^{+/+}$ and $SIT^{-/-}$ lymph node cells. Cells were isolated from $SIT^{+/+}$ (shaded) and $SIT^{-/-}$ (empty) mice and subsequently stained for CD4, CD8 and Bcl-2 expression. The bcl-2 expression profile of gated $CD8^+$ T cells is shown. The dotted line indicates corresponding isotype staining control. C) $CD8^+$ T-cell apoptosis. Ex vivo isolated lymph nodes and spleen cells were stained with CD8 antibody and subsequently with Annexin-V. Mean±Standard Error of the Mean values (SEM) are shown in each graph.

2.4.4. Comparable short-term steady-state proliferation between $SIT^{+/+}$ and $SIT^{-/-}$ T cells

A possible slower steady-state proliferation could also account for the decreased number of $CD8^+$ T cells in SIT-deficient mice. In order to check this possibility, I performed BrdU incorporation assays. Mice were fed with 0.8mg/ml BrdU in their drinking water for 7 days. Then, single cell suspensions from both spleen and lymph nodes were prepared and stained for the expression of CD4, CD8 and BrdU incorporation. As depicted in figure 18, I found no differences in the rate of BrdU incorporation between $SIT^{-/-}CD8^+$ and $SIT^{+/+}CD8^+$ T cells, thus demonstrating that the short-term steady-state proliferation of $CD8^+$ T cells is normal in $SIT^{-/-}$ mice.

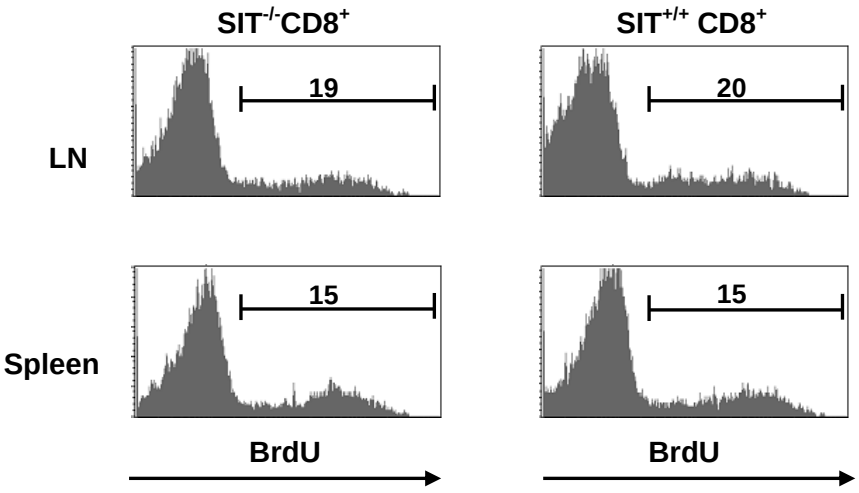


Figure 18. Normal steady-state proliferation of $SIT^{-/-}$ $CD8^+$ T-cells

Lymph node and spleen cells were harvested from BrdU treated (7 days) wild-type and SIT knockout mice and stained with CD4 and CD8 antibodies. Subsequently cells were fixed, permeabilized and further stained intracellulary with an anti-BrdU antibody. Representative BrdU incorporation profiles of $CD8^+$-gated lymph node or spleen T cells are shown. Numbers indicate percentages of cells.

2.5. Altered T-cell homeostasis in SIT-deficient mice

2.5.1. $CD8^+$ peripheral T cells of $SIT^{-/-}$ mice are almost exclusively $CD44^{hi}$

Further investigations of peripheral lymphoid organs also revealed that SIT-deficient mice had a strongly increased proportion of $CD8^+CD44^{hi}$ cells both in lymph nodes and spleen, whereas $CD4^+$ T cells were much less affected (Figure 19). These data indicate that $CD8^+$ T-cell homeostasis is altered in SIT-deficient mice, that results in a bias of $CD8^+$ T cells toward a memory-like phenotype.

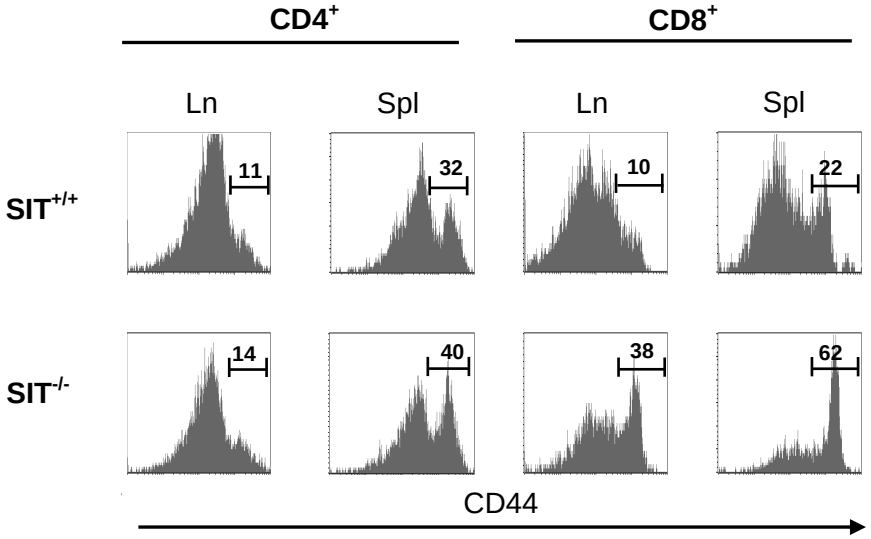


Figure 19. $CD8^+$ T lymphocytes from $SIT^{-/-}$ mice consist mainly of $CD44^{high}$ cells

Single cell suspensions of spleen and lymph nodes from $SIT^{+/+}$ and $SIT^{-/-}$ mice were prepared and stained for the expression of CD4, CD8, and CD44. Here, the CD44 expression profile of gated $SIT^{-/-}$ $CD4^+$ and $CD8^+$ T cells is shown. Numbers indicate percentages in each region.

2.5.2. SIT-deficient $CD8^+CD44^{high}$ cells show a bimodal surface profile

Phenotypically, T cells undergoing homeostatic proliferation are memory-like cells with a bimodal profile, as they express markers of both naïve and memory cells. Based on my previous data loss of SIT seemed to result in an altered peripheral T-cell homeostasis. To shed further light onto this phenomenon, I performed a more detailed FACS analysis of $CD8^+CD44^{high}$ cells from both lymph node and spleen of $SIT^{-/-}$ mice. These studies revealed that SIT-deficient mice had an increased proportion of $CD8^+CD44^{high}CD122^{high}CD62L^{high}$ cells compared with wild-type counterparts (Figure 20A). In addition, both $SIT^{+/+}$ and $SIT^{-/-}$ $CD8^+CD44^{high}$ cells

expressed comparable low levels of the activation marker CD25 (IL-2Rα) (Figure 20C). This profile ($CD8^+CD44^{high}CD122^{high}CD62L^{high}$) observed in $SIT^{-/-}$ mice is identical to the profile of T cells undergoing homeostatic proliferation. This observation further indicates that $CD8^+CD44^{high}CD122^{high}CD62L^{high}$ $SIT^{-/-}$ T cells are not true antigen experienced memory but rather memory-like cells generated by an altered peripheral T-cell homeostasis.

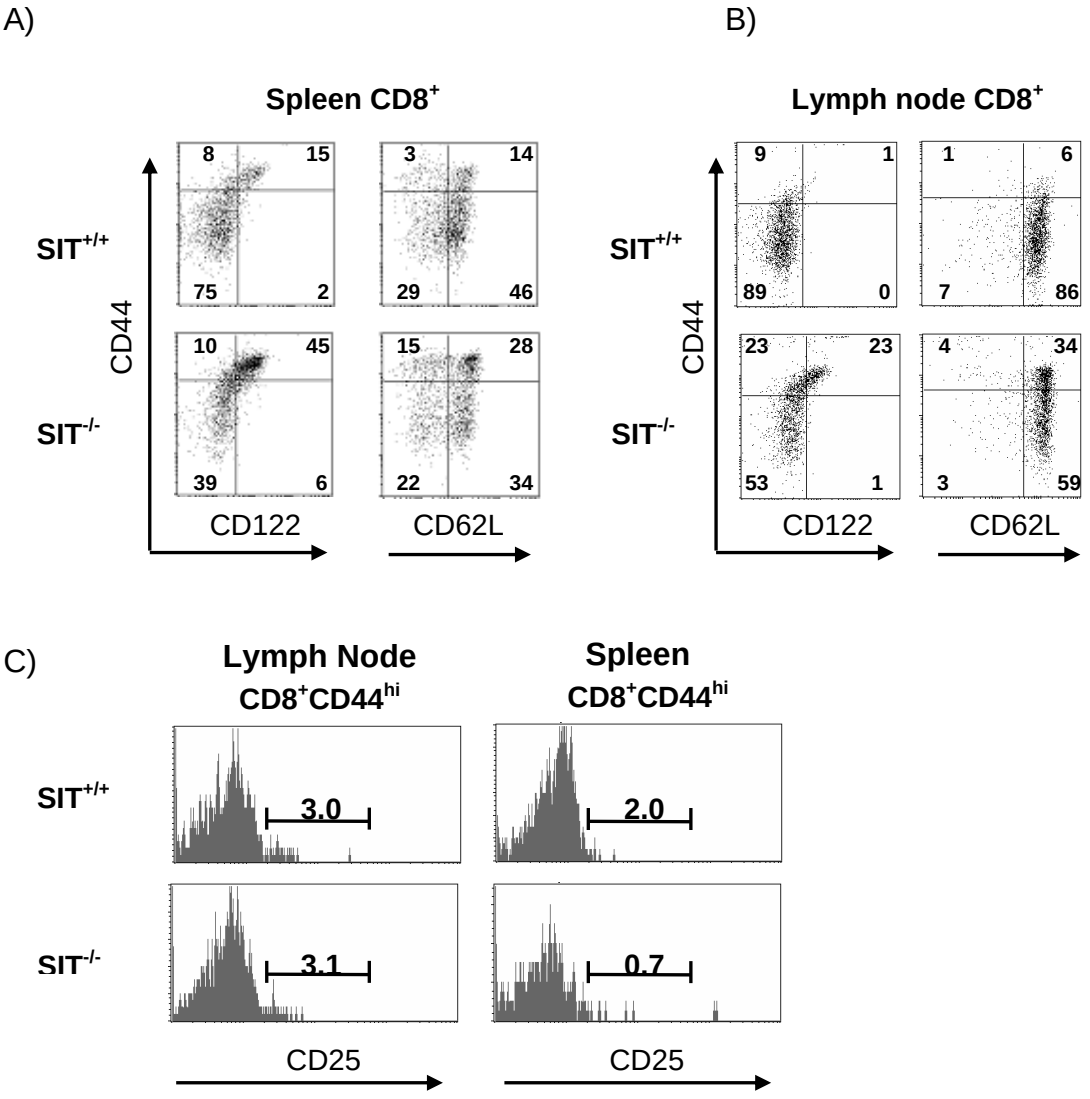


Figure 20. Bimodal profile of $SIT^{-/-}CD44^{hi}CD8^+$ T cells

Representative CD44, CD122 and CD62L expression profile of gated $CD8^+$ T cells from spleen (A) and lymph nodes (B). Single cell suspensions from both spleen and lymph nodes were prepared and subsequently stained with CD44, CD122, CD62L antibodies. C) Single cell suspensions were prepared from both lymph nodes and spleens of $SIT^{+/+}$ and $SIT^{-/-}$ mice. Subsequently, cells were stained with CD4, CD8, CD44 and CD25 antibodies. CD25 expression of gated $CD8^+CD44^{hi}$ T cells is shown. Numbers in each quadrant indicate percentages.

2.5.3. Elevated CD5 expression in SIT$^{-/-}$ CD8^{+} T cells

Peripheral T cells require signals generated by TCR:self-peptide-MHC interactions to survive and to undergo slow rate homeostatic proliferation. It is also worth mentioning that T cells carrying TCRs with stronger affinity have proliferation advantage in lymphopenic hosts as compared to cells expressing TCRs with lower affinity (48). Thus, an enhanced TCR-mediated signalling might also result in an enhanced homeostatic proliferation. To this end, I tested whether loss of SIT resulted in an increased TCR affinity in peripheral CD8^{+} T cells. Similar to thymocytes, also peripheral CD8^{+} T cells showed a significant upregulation of CD5 indicating that T cells in SIT-deficient mice indeed receive a permanently stronger signal via the TCR that might result in an altered T-cell homeostasis (Figure 21, see also Figure 29A).

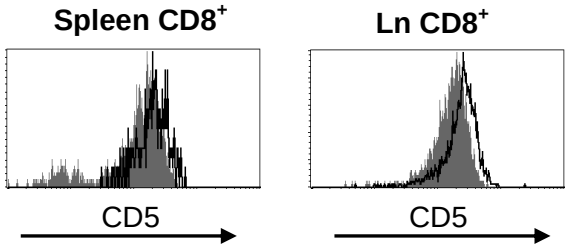


Figure 21. Elevated CD5 expression level on SIT-deficient CD8^{+} T-cells

Expression of CD5 on gated CD8^{+} T-cells. Lymph node and spleen cells were stained with CD8 and CD5 antibodies. Overlayed histograms show CD5 expression on CD8^{+}-gated SIT$^{+/+}$ (shaded) and SIT$^{-/-}$ (empty) T-cells.

2.5.4. SIT$^{-/-}$ mice accumulate CD8^{+}CD44^{hi} T cells in secondary lymphoid organs

We hypothesized that, SIT$^{-/-}$CD8^{+} T cells acquire a memory-like phenotype upon enhanced homeostatic expansion, that would result in a corresponding accumulation of CD44^{high} T cells in the periphery. Indeed, SIT-deficient mice progressively accumulated memory-like T cells in the spleen over the time as compared to wild-type controls. This indicates that T-cell homeostasis is altered in SIT$^{-/-}$ mice (Figure 22).

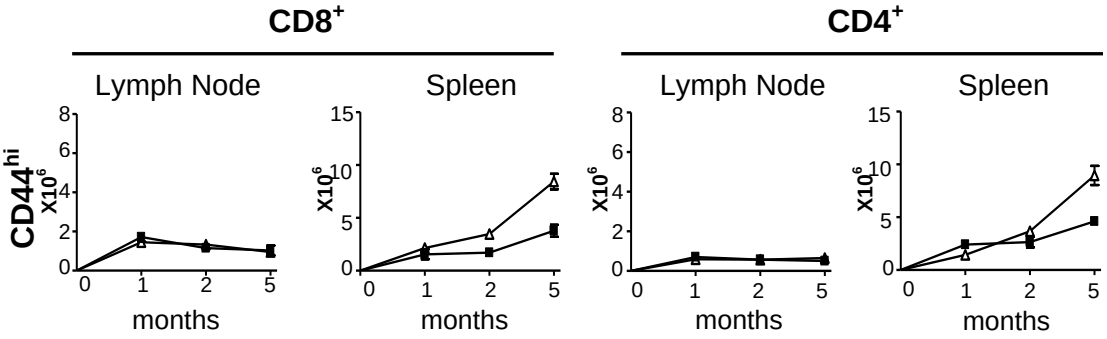


Figure 22. Progressive accumulation of CD8^{+}CD44^{high} cells in SIT-deficient mice

Absolute numbers of CD8^{+}CD44^{high} and CD4^{+}CD44^{high} T cells. Based on CD44 expression absolute numbers of CD44^{low} and CD44^{high} cells were calculated within both CD4^{+} and CD8^{+} T-cell subsets. Means ±Standard Error of the Mean values (SEM) are shown in each graph. Empty triangles indicate SIT-knockout whereas filled squares represent wild-type mice.

2.6. SIT regulates homeostatic proliferation of MHC-I-restricted TCR transgenic T cells

2.6.1. Loss of SIT allows HY tg CD8^{+} T cells to undergo homeostatic proliferation in irradiated hosts

To directly test the role of SIT in CD8^{+}T-cell homeostasis, I adoptively transferred transgenic CD8^{+} T cells expressing TCRs with different affinities (HY, P14, OT-I) into irradiated recipients. Given the remarkable influence of SIT on positive selection in HY transgenic mice, first I decided to test whether SIT has also a strong influence on the homeostatic behaviour of peripheral HY T cells. Strikingly, I found that SIT$^{-/-}$ HYCD8^{+} transgenic cells have undergone homeostatic expansion in lymphopenic hosts, whereas as reported SIT$^{+/+}$ counterparts failed to do so (Figure 23). Similarly, loss of SIT resulted in an enhanced homeostatic proliferation also of P14 tg CD8^{+} T cells. Finally, I wished to test the homeostatic behaviour of OT-I CD8^{+} T cells that possess TCR with strong affinity. However, loss of SIT did not result in any alteration

in the homeostatic proliferation capacity of OT-I $CD8^+$ T cells in lymphopenic hosts (Figure 23). This is similar to the situation found in the thymus where thymic selection was also unaffected. Thus, SIT regulates T-cell homeostasis and development in a strikingly similar manner. In conclusion, the ability of SIT to regulate TCR-mediated signalling is restricted to TCRs with low-intermediate affinities.

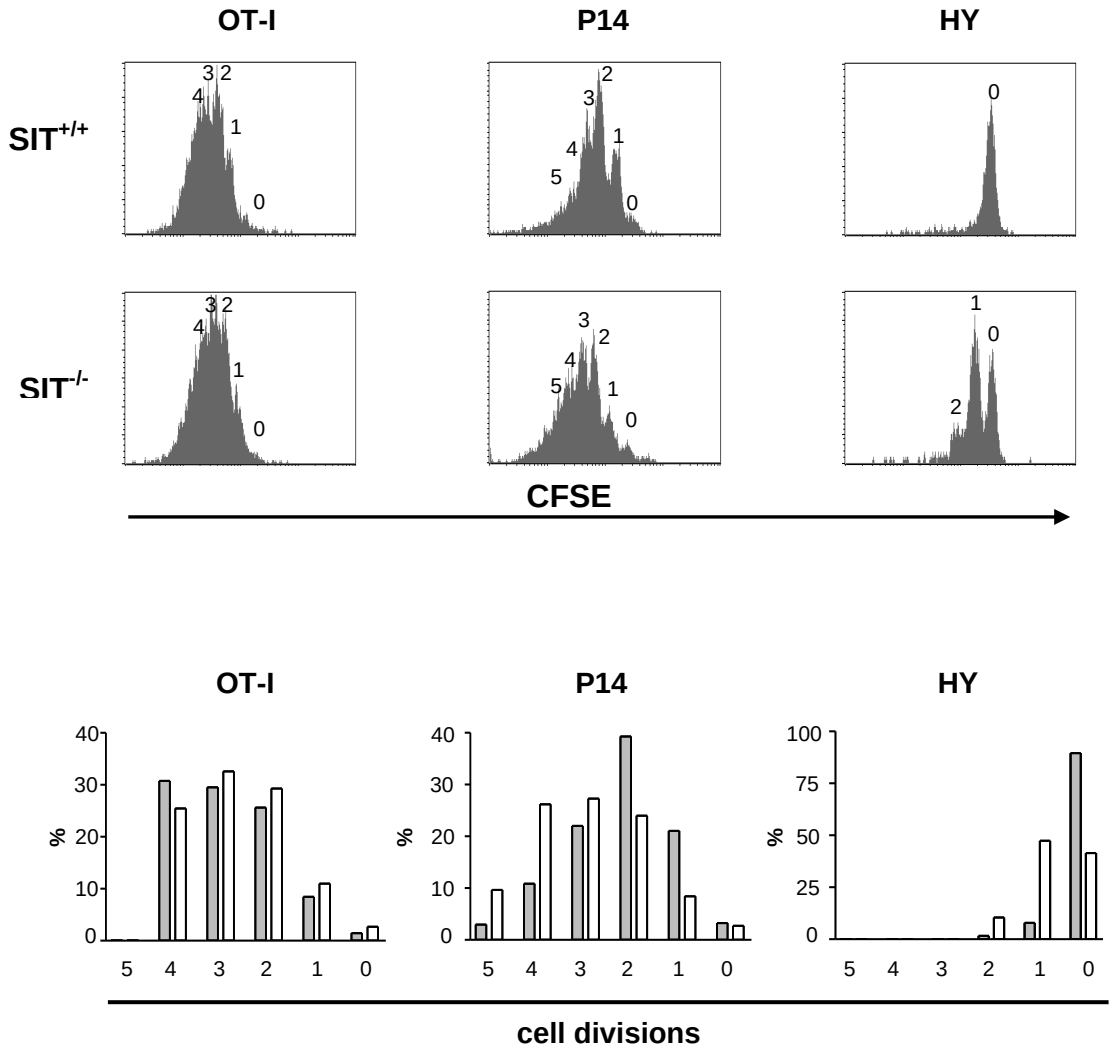


Figure 23. SIT functions as a potent negative regulator of homeostatic proliferation of CD8$^+$ T cells

Homeostatic proliferation of adoptively transferred MHC-I restricted CD8$^+$ transgenic T cells. A representative profile of multiple experiments is shown. CFSE-labelled lymph node cells from HY, P14 or OT-I SIT$^{+/+}$CD45.2$^+$or SIT$^{-/-}$CD45.2$^+$ transgenic mice were adoptively transferred into lethally irradiated CD45.1$^+$ recipients. After seven (HY, P14) or 3 days (OT-I) cells were harvested and CFSE profiles of gated donor derived (CD45.1$^-$) CD8$^+$ T cells were analysed. Numbers shown above peaks indicate cell divisions. Histograms shown here represent four P14, four OT-I and at least six HY mice per each genotype. Gray bars indicate wild-type whereas empty bars represent SIT$^{-/-}$ mice.

2.6.2. SIT$^{-/-}$ HY transgenic CD8^{+} T cells undergo homeostatic proliferation in rag1$^{-/-}$ hosts

As a side effect of irradiation a strong cytokine storm is reported to occur (40, 84). The altered cytokine environment induced by irradiation might have induced homeostatic expansion of SIT$^{-/-}$ HY cells. To rule out the possibility that the striking proliferation of SIT$^{-/-}$ HY transgenic cells was induced by an abnormal cytokine release triggered by irradiation, I injected CFSE-loaded LN cells from HY female mice into female Rag1$^{-/-}$ mice. Peripheral lymphoid organs of rag1$^{-/-}$ deficient mice have a more physiological cytokine environment as compared to irradiated mice. As shown in figure 24, similar to irradiated recipients SIT$^{-/-}$HYCD8^{+} T cells have undergone the same number of divisions also in rag1$^{-/-}$ hosts. Thus, we could exclude the possibility that the proliferation of SIT$^{-/-}$HY^{+}CD8^{+} T cells observed in irradiated hosts was due to an irradiation side effect.

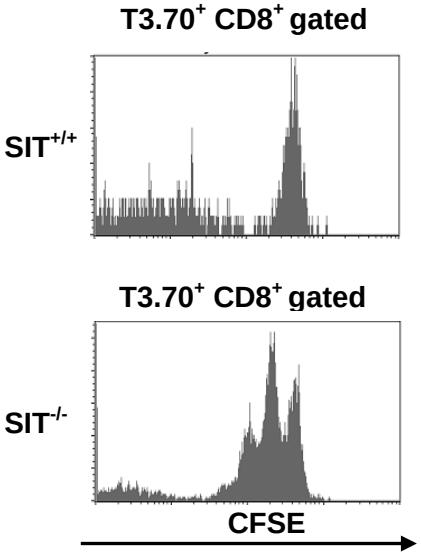


Figure 24. HY TCR tg T cells undergo homeostatic proliferation in rag1$^{-/-}$ mice

Homeostatic proliferation of adoptively transferred HY transgenic CD8^{+} T cells. CFSE- labelled lymph node cells from HY CD45.2^{+} transgenic mice were adoptively transferred into lethally irradiated CD45.1^{+} recipients. After 7 days, injected cells were recovered and CFSE profiles of gated donor derived (CD45.1^{-}) T3.70^{+} CD8^{+} T cells were analysed. Histograms shown here represent results of multiple experiments.

2.7. SIT$^{-/-}$ and SIT$^{+/+}$ CD8^{+} T cells responded equally to cytokines in vitro

Cytokines, mainly IL-7 and IL-15 play a major role in regulating homeostatic proliferation. To rule out the possibility that a different sensitivity to cytokines was responsible for the altered T-cell homeostasis in SIT-deficient mice, I investigated the ability of SIT$^{+/+}$ and SIT$^{-/-}$ T cells to respond to cytokines *in vitro*. Total lymph node cells form SIT$^{-/-}$ and SIT$^{+/+}$ mice were cultured with or without cytokines for three days. As expected, IL-7 induced no proliferation of SIT$^{+/+}$ and SIT$^{-/-}$ CD8^{+} T cells. Conversely, IL-15 induced a strong proliferation of CD8^{+} T cells. Nonetheless, I found no difference in the number of cell cycles induced by IL-15 between SIT$^{+/+}$ and SIT$^{-/-}$ CD8^{+} T cells. Remarkably, quantitatively more CD8^{+} T cells responded to *in vitro* cytokine stimuli (Figure 25). This effect is most likely due to the higher proportion of CD44^{high} cells within the CD8^{+} T cell subset in SIT-deficient mice (discussed above).

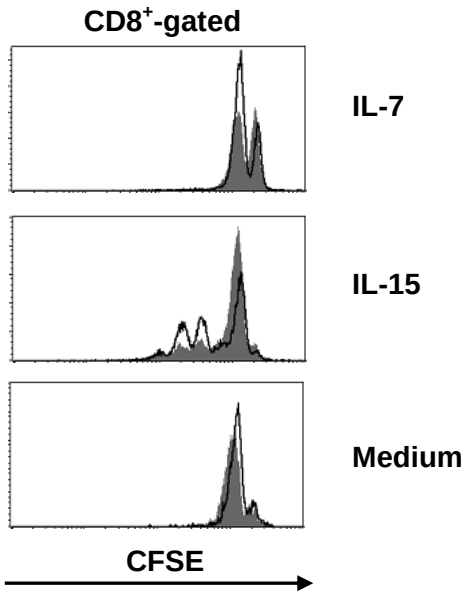


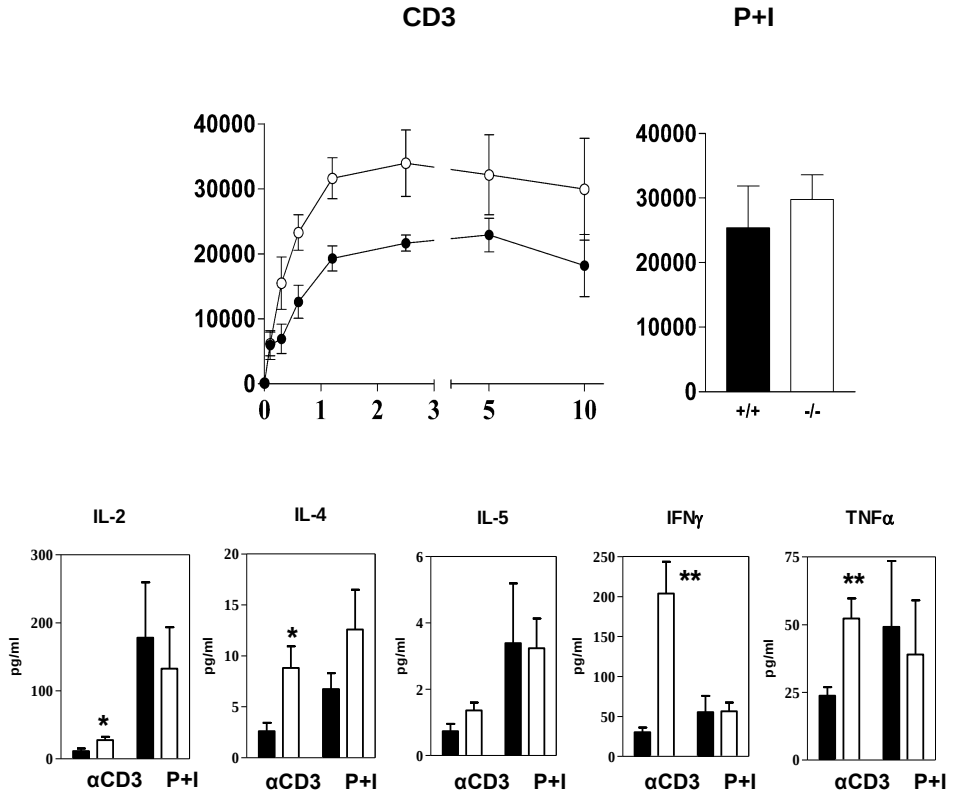
Figure 25. SIT$^{-/-}$ CD8^{+} T cells show normal proliferation upon by cytokine stimulation *in vitro*

Lymph node cells were isolated, loaded with CFSE and subsequently cultured for three days with cytokines (IL-7, IL-15) or left unstimulated. After three days, cells were harvested and stained with CD8 antibody and subsequently analysed by flow cytometry. Representative overlayed CFSE profiles of CD8^{+}-gated SIT$^{+/+}$ (shaded) and SIT$^{-/-}$ (empty) T cells are shown.

2.8. SIT$^{-/-}$ T cells are hyperresponsive to TCR stimulation in vitro

2.8.1. Enhanced proliferation of SIT-deficient T cells *in vitro*

So far, I have shown that loss of SIT affected T-cell development and homeostasis *in vivo*. Next, I tested whether loss of SIT also affected T-cell function *in vitro*. To this end, I performed *in vitro* proliferation assays. Purified T cells derived either from SIT$^{+/+}$ or from SIT$^{-/-}$ mice were stimulated with increasing concentrations of plate-bound CD3 antibody. After 72 hours, cells were harvested and subsequently [^{3}H] thymidyne incorporation was determined. Figure 26A shows that SIT$^{-/-}$ T cells proliferated stronger especially when stimulated with suboptimal concentration of CD3. However, when stimulated with PMA both SIT$^{+/+}$ and SIT$^{-/-}$ T cells showed equal proliferation. This indicates that SIT$^{-/-}$ T cells signal normally downstream of PKC (Figure 26A). In addition, upon CD3 stimulation SIT$^{-/-}$ T cells showed an elevated release of TH1 (T-helper 1) type cytokines indicating that absence of SIT skews peripheral T cells toward TH1 subset (Figure 26B).



A)

Figure 26. SIT$^{-/-}$ T cells are hyperreactive and show elevated cytokine release

A) Enhanced proliferation of SIT-deficient T cells. Purified splenic T cells were stimulated with plate-bound CD3 antibody or with PMA and ionomycin (bar graph) in 96-well plates. Cells were pulsed with [^{3}H]-thymidine and processed for standard scintillation counting. Graphs indicate results obtained from seven wild-type and nine SIT-knockout mice. Mean values are shown. One of two independent experiments is shown. p=0.01(*),P=0.007(**). B) Enhanced production of cytokines upon CD3 stimulation. Purified T cells were stimulated either with plate- bound CD3 (0,3ug) or with PMA+ionomycin (P+I). Cytokine levels were determined from supernatants of stimulated cells. Results are obtained from eight wild-type and ten SIT-knockout mice. p=0.02(*), p<0.005(**). Black bars represent wild-type mice whereas empty bars indicate SIT-deficient mice.

B)

2.8.2. SIT-deficient mice develop a more severe EAE

SIT-deficient T cells have a lowered threshold of activation, an altered homeostasis, and are hyperreactive to TCR-mediated stimulation *in vitro.* Therefore, I wished to investigate whether SIT-deficient mice were more susceptible to develop autoimmune diseases. To this end, in collaboration with Dr. Dirk Reinhold, we used EAE (Experimental Autoimmune Enchephalitis) a well-established model of multiple sclerosis. As expected $SIT^{-/-}$ mice develop a faster as well as more severe EAE (Figure 27)**.** In addition, aged SIT-knockout mice developed a spontaneous Lupus-like disease and displayed an accumulation of activated $CD4^+$ T cells in secondary lymphoid organs (Börge Arndt, et al. manuscript in preparation). Together, these data indicate that loss of SIT resulted in an enhanced susceptibility to develop autoimmunity.

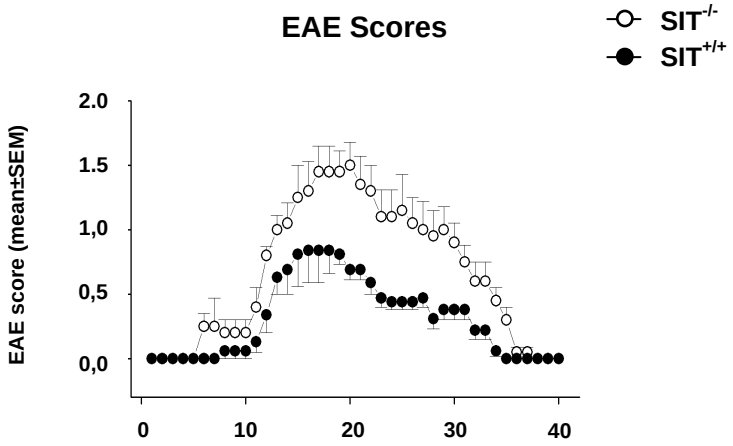


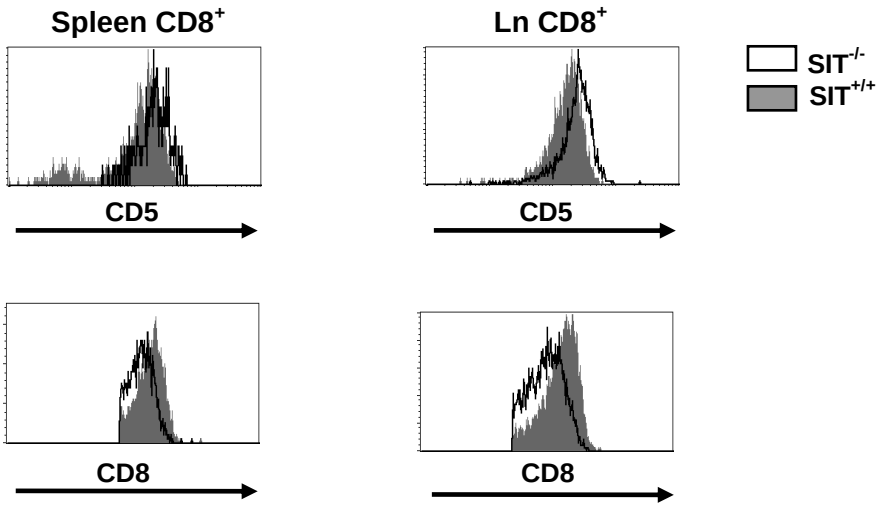
Figure 27. $SIT^{-/-}$ mice develop a more severe EAE

EAE scores of $SIT^{+/+}$ and $SIT^{-/-}$ mice. Mice were examined daily for the signs of EAE and graded on a scale of increasing severity from 0 to 5 as follows: 0 no signs, 0.5 partial tail weakness, 1 limp tail or slight slowing of righting from supine position, 1.5 limp and tail and slight slowing of righting, 2, partial hind limb weakness or marked slowing of righting, 2.5 dragging of hind limb(s), without complete paralysis, 3, complete paralysis of at least one hind limb, 3.5 hind limb paralysis and slight weakness of forelimbs, 4 severe forelimb paralysis, 5 moribund or death. EAE experiments and scoring were performed by Dr. Dirk Reinhold.

2.9. Sensory adaptation in SIT$^{-/-}$ CD8^{+} T cells

Downregulation of CD8 is a mechanism that allows T cells to regulate the activation threshold. This could be required to counterbalance the effects of chronic stimulation (59). As SIT-deficient T cells are hyperactivated they likely develop compensatory mechanisms similar to sensory adaptation that prevents them from becoming fully activated. This mechanism could help to maintain an efficient and controllable immune response. Indeed, when challenged with Toxoplasma or Listeria, SIT knockout mice showed a normal immune response. In addition, SIT-deficient mice showed a normal allograft rejection (unpublished observations from our laboratory). To test whether SIT$^{-/-}$ T cells develop sensory adaptation I analysed the expression level of both CD4 and CD8 coreceptors. Indeed, I found a significant downregulation of the CD8 coreceptor already in SIT$^{-/-}$ mice (Figure 28A). In agreement with this observation, a marked CD8 coreceptor downregulation was also observed in both HY and P14 transgenic mice. Downregulation of the CD8 resulted in accumulation of CD4^{-}CD8^{-} TCR transgenic T cells in the periphery of TCR transgenic HY and P14 mice (Figure 28B). Additionally, SIT$^{-/-}$CD8^{+} T cells express higher levels of CD5 (Figure 28A and discussed above) a potent negative regulator of TCR-mediated signalling. Taken together, SIT$^{-/-}$ mice developed a mechanism similar to sensory adaptation in order to dampen TCR mediated signalling intensity. This mechanism likely in cooperation with others, prevents CD8^{+} T cells lacking SIT from becoming fully activated, thus allowing SIT-deficient mice to mount a controlled immune response when challenged with pathogens. However, the compensatory mechanism is only partially successful since SIT mice are more susceptible to EAE (see above) as well as develop activated CD4^{+} T cells at the age of one year (Börge Arndt et al., manuscript in preparation).

A)



	SIT+/+	SIT-/-	
CD5 MFI	165.6 ± 4.27	230.2 ±6.86	p< 0.0001 n=8
CD8 MFI	189.8 ± 6.3	121.5 ± 6.55	p< 0.0001 n=8

	SIT+/+	SIT-/-	
CD5 MFI	177.5 ±7.8	245.3 ± 7.0	p< 0.0001 n=8
CD8 MFI	166.2 ± 20.46	120.5 ± 3.41	p<0.0033 n=8

B)

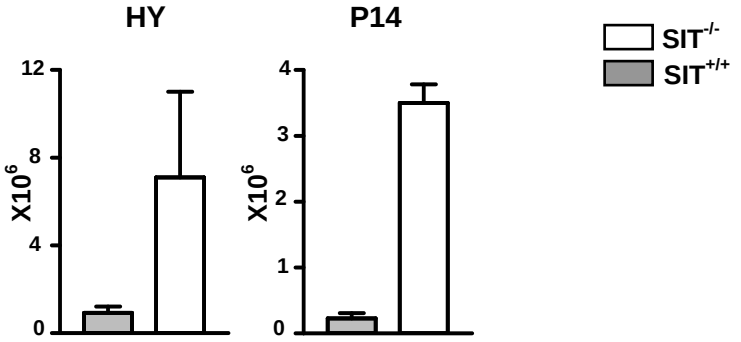


Figure 28. Sensory adaptation in SIT$^{-/-}$CD8^{+} T cells

A) Expression of CD5 and CD8 on WT and SIT-deficient CD8^{+} T cells. Splenocytes were prepared and stained with CD5 and CD8 antibodies. Subsequently, flow cytometric analysis was carried out. The expression of CD5 (upper panel) and CD8 (lower panel) on CD8^{+}-gated T cells is shown. Table below indicates corresponding CD5 and CD8 MFI (mean fluorescence intensity) values. Statistical analyses were performed by using data obtained from 8 wild-type and 8 knockout mice. B) CD8 coreceptor downregulation in HY and P14 mice. TCR transgenic mice were stained with CD4, CD8, and TCRα (T3.70^{+} for HY and Vα2^{+} for P14) antibodies. Numbers of transgenic TCRα^{+}CD4^{-}CD8^{-} cells were calculated.

2.10. TCR-proximal signalling is impaired in SIT$^{-/-}$ mice at the level of Zap70 activation

I next investigated TCR proximal signalling events in SIT$^{-/-}$ thymocytes and T cells. Thymocytes from SIT$^{+/+}$ and SIT$^{-/-}$ mice were stimulated with crosslinked CD3 antibody and subsequently lysed at the the indicated time points. Lysates were loaded on SDS PAGE and Western blot analysis was carried out. To assess global tyrosine phosphorylation in stimulated SIT$^{-/-}$ T cells, membranes were probed with anti p-Tyr (phospho-tyrosine) antibody. Surprisingly, we observed a general reduction of total Tyr phosphorylation in SIT$^{-/-}$ T cells. Strikingly, the phosphorylation of LAT (pp36-38) (Figure 29) was also severely impaired. Since LAT is known to be the substrate of the tyrosine kinase Zap-70, we have investigated whether Zap70 activation was also impaired in SIT-deficient T cells. To this end, wild-type and SIT-deficient thymocytes were stimulated with CD3 antibody, lysed and subsequently SDS PAGE was carried out. As figure 29 shows, Zap-70 phosphorylation as well as its kinase activity were strongly reduced in SIT$^{-/-}$ thymocytes. To further assess whether the severely impaired proximal signalling has any effect on more downstream signalling pathways such as MAPK activation we have also tested the level of phosphorylated ERK in SIT$^{+/+}$ and SIT$^{-/-}$ thymocytes. As figure 29 shows, the level of phosphorylated ERK was reduced in SIT$^{-/-}$ thymocytes as compared to wild-type cells whereas JNK and P38 activation remained unaffected.

Thus, proximal signalling events are severely impaired in SIT-deficient thymocytes. Moreover, further investigations revealed that similar to thymocytes, SIT-deficient peripheral T cells also show the same alteration of TCR-proximal signalling (Figure 30). These data clearly indicate a presence of a strong compensatory mechanism(s) in SIT$^{-/-}$ T cells.

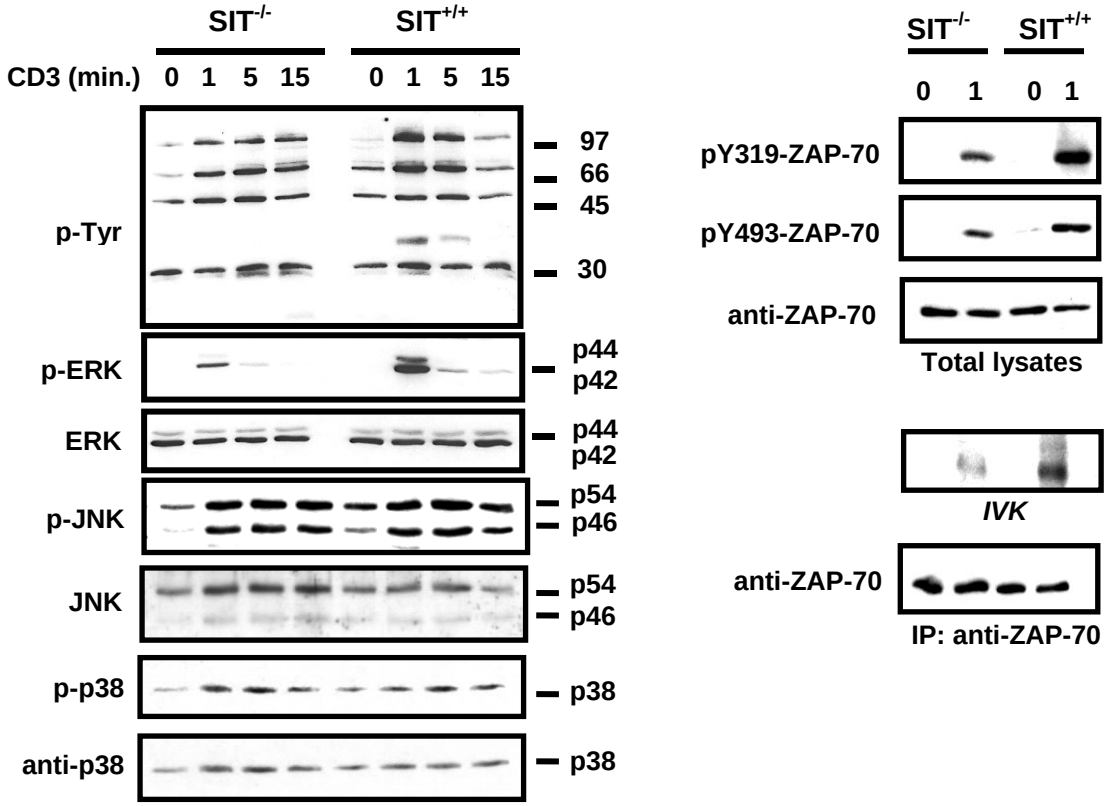


Figure 29. Reduced proximal signalling in SIT-deficient thymocytes

Thymocytes were stimulated for the indicated times and assayed for the induction of total tyrosine phosphorylation (p-Tyr) or the activation of ERK1/2, JNK and p38. B) Thymocytes were stimulated and processed for immunoblotting. Phosphorylation of ZAP70 was analyzed by immunoblotting total post-nuclear cell lysates directly. ZAP70 immunoprecipitates were subjected to in vitro kinase assay. Experiment shown here was performed by Ines Meinert and Dr. Luca Simeoni.

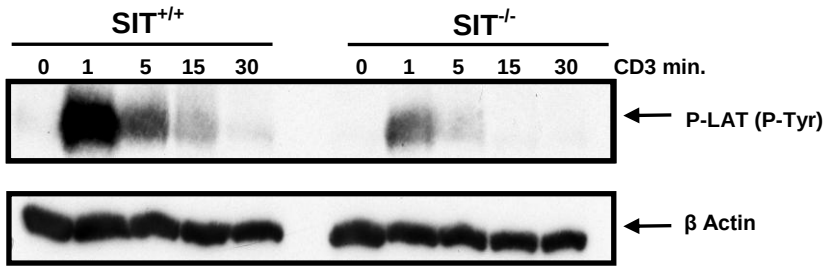


Figure 30. Reduced LAT phosphorylation upon CD3 stimulation of SIT$^{-/-}$ T cells

Phosphorylation of LAT upon CD3 stimulation. A representative Western blot of three independent experiments is shown. Purified total T cells from spleen were stimulated with CD3 antibodies or left unstimulated. Subsequently, cells were lysed and SDS-PAGE was carried out. Membranes were probed with p-Tyr (clone: 4G10) antibody. A phosphoprotein of 36-38 kDa protein corresponding to LAT is shown. Equal protein loading was assessed by reprobing the membrane with β-actin antibody.

Discussion

Transmembrane adaptor proteins are key organizers of TCR-mediated signalling events. They recruit intracellular signalling proteins, thus serving as scaffolds to organize multisignalling complexes. In fact, TRAPs are essential to link the TCR to more downstream signalling events (41). By characterizing SIT-deficient mice, I showed that SIT regulates TCR-mediated processes such as T-cell development and T-cell homeostasis.

SIT was previously shown to negatively regulate TCR-mediated signalling in Jurkat T cells (63, 85). In mice, SIT is expressed most abundantly in the thymus (Figure 6 and (103)). Therefore, I initially investigated the role of SIT in T-cell development. SIT-deficient mice display a higher percentage of DP and a decreased proportion of SP thymocytes. In addition, $SIT^{-/-}$ thymocytes have elevated levels of CD5 as well as CD69 as compared to wild-type cells (Figure 8). The upregulation of these two markers has been shown to correlate with the activation status of thymocytes (6, 7, 73, 110). Thus, SIT-deficient thymocytes display a more activated phenotype (Figures 7-10). Moreover, SIT-deficient HY transgenic thymocytes display a more mature phenotype, a strongly reduced DP compartment and, elevated level of phosphorylated ERK, whose activation directly correlates with positive selection (3, 87). Taken together, the more activated phenotype of SIT-deficient thymocytes, the strongly reduced proportion of DP thymocytes in HY mice, the more matured phenotype as well as the higher level of phosphorlated ERK, collectively demonstrate that loss of SIT results in an enhanced positive selection. It is likely that SIT lowers the activation threshold of thymocytes. In fact, SIT-knockout HY transgenic mice also showed a decreased thymocyte number demonstrating that, besides enhanced positive selection, a partial shift from positive to negative selection takes place in these mice (Figure 10). In agreement with these results, SIT-deficient mice expressing the P14 TCR with higher affinity displayed an almost complete shift from positive to negative selection (Figure 10). Of importance, SIT-deficient HY male mice show an identical thymic cellular composition as compared to wild-type HY male mice ruling out the possibility that SIT would directly influence negative selection (Figure 12). Interestingly, T-cell development of both SIT deficient OT-I and OT-II mice was unaltered (Figure 11). This excludes the possibility that SIT would directly regulate apoptosis of thymocytes or the expression of CD5 or CD69. In addition the unaltered T-cell development in OT-I mice also indicates that SIT acts in a TCR affinity- dependent manner as beyond a certain TCR affinity it becomes dispensable. Moreover, SIT seems to influence mainly $CD8^{+}$ T-cell development. However, the

role of SIT in the development of $CD4^+$ T cells still needs to be further investigated by using other MHC-II restricted transgenic mouse models. Taken together, these results demonstrate that SIT functions as a negative regulator of TCR-mediated signalling *in vivo.* Indeed, mice lacking other negative regulators of TCR-mediated signalling such as CD5 show strikingly similar phenotype to SIT-deficient mice (6, 114). As TRAPs have similar structures, it is likely that other TRAPs may compensate for the loss of SIT. TRIM, another non-raft associated transmembrane adaptor protein, displays structural homology and shares similar TBSMs with SIT (YGNL and YASV/L respectively). The fact that HY transgenic SIT/TRIM double-knockout mice show a complete conversion from positive to negative selection strongly suggests that TRIM is indeed partially redundant with SIT during T-cell development (Uwe Kölsch et al., manuscript in preparation). Unlike TRIM, LAX (another non-raft associated TRAP) that similar to SIT possesses multiple Grb2 binding sites does not seem to compensate for SIT in terms of T-cell development. This conclusion is drawn from the fact that LAX knockout mice do not show alteration of T-cell development and SIT/LAX double-knockout mice display the same alteration described in SIT-deficient mice (Börge Arndt et al., manuscript in preparation). Thus, among non-raft associated TRAPs, SIT and TRIM seem to be major regulators of T-cell development.

SIT is also strongly expressed by peripheral T cells (Figure 6 and (103)). Based on its strong expression as well as its ability to set the threshold for thymocyte activation, we hypothesized that SIT would also influence peripheral T-cell functions. Investigation of peripheral lymphoid organs revealed that SIT-deficient mice have a reduced lymph node cellularity (Figure 14-15). More importantly, SIT-deficient mice progressively accumulate memory-like $CD8^+$ T cells and display a reduction in the number of naïve $CD8^+$ T cells both in lymph nodes and spleen (Figure 22). Interestingly, five month old SIT-deficient mice also accumulate memory-like $CD4^+$ T cells. However, the $CD4^+$ naïve T cell pool is unaltered in these mice. Thus, the $CD8^+$ T-cell pool is more affected by the loss of SIT. The fact that SIT-deficient $CD8^+$ T cells show comparable level of apoptosis, normal steady state proliferation as well as Bcl-2 expression (whose expression is crucial for T-cell survival) (14, 49, 72, 117) rules out the possibility that loss of SIT would result in an intrinsic survival or proliferation defect. We propose that SIT contributes to the maintenance of the peripheral T-cell pool, by controlling other mechanisms such as the threshold of T-cell activation, generation of naïve T-cell precursors and T-cell homeostasis. SIT-knockout mice display a reduced proportion of $CD103^+$ $CD8^+$ T cells (Figure 16). CD103 has recently been reported as a marker of T cells with direct thymic origin

termed as recent thymic emigrants (65, 106). This result shows that SIT regulates the frequency of naïve T-cell precursors. Moreover, the progressive accumulation of memory-like T cells suggests that the slow rate peripheral T-cell homeostasis is also strongly altered by the loss of SIT. Peripheral T-cell homeostasis is responsible for maintaining constant peripheral T cell number during the course of life. This process is strongly dependent on low-affinity TCR:self-peptide-MHC interactions (13, 30, 47, 64, 69, 77, 111, 113). Homeostatic proliferation is distinct from antigen driven proliferation since does not require costimulation. Moreover, T cells undergoing homeostatic expansion do not upregulate activation markers such as CD25 or CD69. It has been also shown that low-affinity peptides that mediate positive selection are also involved in controlling T-cell survival and homeostasis (30). Indeed, TCR transgenic mice possessing TCRs with different affinities display different abilities to undergo homeostatic proliferation when injected into lymphopenic hosts. In fact, T cells expressing TCR with a strong affinity have proliferative advantage over those expressing low-affinity TCRs (Figure 23 and (30, 48)). In line with these observations, HY transgenic T cells that possess low-affinity TCR are reported not to undergo homeostatic proliferation upon adoptive transfer into lymphopenic hosts (30, 35, 48). Strikingly, SIT-deficient HY transgenic $CD8^+$ T cells do undergo lymphopenia-induced proliferation. Thus, loss of SIT resulted in the homeostatic expansion of HY tg T cells. Based on the same principle SIT deficiency resulted in a more pronounced homeostatic expansion of P14 transgenic T cells, thus SIT regulates TCR-mediated signalling strength (Figure 23).

In summary, these results strongly and clearly demonstrate that SIT regulates both T-cell development and homeostasis in a strikingly similar manner. Other molecules such as BTLA or LAG3 have also been reported to regulate T-cell homeostasis without strongly altering the activation status of T cells. However, SIT is the only molecule that has been so far shown to regulate both T-cell development and homeostasis in a strikingly similar fashion (52, 121). $CD8^+$ T cells are more affected by the loss of SIT than $CD4^+$ T cells. This observation can be explained by the fact that the homeostasis of $CD8^+$ T cells is differentially regulated compared to those of $CD4^+$ T cells. The fact that the adaptor protein GADS that is expressed in both $CD8^+$ and $CD4^+$ T cells has been also shown to selectively regulate $CD4^+$ but not $CD8^+$ T-cell homeostasis further supports this view (124).

Collectively, our results demonstrate that SIT contributes to the maintenance of peripheral T-cell pools by at least two mechanisms. First, by regulating the number of T-cell precursors and second, by controlling peripheral T-cell homeostasis. The strong influence of SIT on both T-cell homeostasis and development is based on its ability to negatively regulate TCR-mediated signalling. Indeed, T cells from SIT deficient non-transgenic mice are hyperreactive upon TCR triggering *in vitro* (Figure 27). In agreement with this observation, they also express higher levels of CD5, thus indicating that they permanently receive a stronger signal via the TCR (Figure 21). Based on our data, we propose that low affinity TCR: self-peptide-MHC mediated signals that usually promote T-cell survival become overtly stimulatory and induce a continuous low rate homeostatic expansion of T cells in SIT-deficient mice. During this expansion phase T cells would gradually acquire a memory-like phenotype. This mechanism together with the reduced numbers of T-cell precursors would also explain the decreased numbers of naïve $CD8^+$ T cells.

SIT-deficient T cells display both lowered thresholds of activation and impaired homeostasis. However, young SIT-deficient mice do not develop chronic inflammatory diseases. Hence, we propose the presence of compensatory mechanism(s) that prevent SIT-deficient T cells from becoming fully activated. Indeed, SIT-deficient T cells display signs similar to sensory adaptation such as CD8 coreceptor downregulation or upregulation of CD5 (Figure 29 and (6, 7, 59, 104)). The strongly enhanced proportion of double negative T cells observed in both HY and P14 transgenic SIT-deficient mice further demonstrates the presence of sensory adaptation mechanisms induced by SIT-deficiency (Figure 28). Indeed, when investigated at the molecular level SIT-deficient thymocytes showed an impaired Zap70 kinase activity (Figure 29). Most likely a similar compensation mechanism is present in peripheral T cells as indicated by the strongly impaired LAT phosphorylation upon TCR triggering *in vitro* (Figure 30). However, the compensatory mechanism(s) seem(s) to be only partially efficient since aged $SIT^{-/-}$ mice develop a Lupus-like disease. (Börge Arndt et al, manuscript in preparation).

SIT possesses five tyrosine-based signalling motifs that were shown to become phosphorylated upon TCR triggering in Jurkat T cells. Upon phosphorylation, TBSMs of SIT recruit molecules such as Grb2, SHP2, and Csk that are capable of regulating various intracellular signalling pathways (63, 85). Though upon TCR stimulation SIT recruits SHP2 via its ITIM motif, yet the negative regulatory effect of SIT in Jurkat cells is exclusively mediated via the YASV motif (63, 85). However, it can be that SIT functions differentially in primary mouse T cells. Thus, one has to consider that,

unlike Jurkat T cells where SIT might exert its negative regulatory function via the YASV motif, in primary mouse T cells the negative regulatory effect of SIT could be mediated via the ITIM motif and therefore could involve SHP-2. This view is supported by the fact that mice lacking other negative regulatory molecules possessing ITIM motifs such as CD5, PD-1 and BTLA display a simiar phenotype to $SIT^{-/-}$ mice. Another possible mechanism is that SIT, by recruiting Grb2 upon TCR engagement, would sequester away this molecule from lipid rafts. Thus, the reduced availability of Grb2 would result in a reduced TCR mediated signalling strength. Detailed investigation of SIT-deficient mice have revealed that SIT is a potent regulator of multiple aspects of T-cell functions such as development, threshold of activation and homeostasis. These results highlight the role of TRAPs in regulating a wide spectrum of immune functions with clinical relevance.

Abbreviations

aa	amino acid
BSA	bovine serum albumin
Csk	C-terminal Src kinase
DMSO	dimethylsulfoxide
DN	double negative
DP	double positive
EAE	experimental aoutimmune encephalomyelitis
EDTA	ethylenediamine tetraacetic acid
ERK	extracellular signal-regulated protein kinase
FCS	fetal calf serum
FITC	flourescein isotyocyanate
Gads	Grb2 related adapter protein
GEM	glycosphyngolipid-enriched microdomains
Grb2	growth factor receptor binding protein 2
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
LAT	linker for activation of T cells
LAX	linker for activation of X cells
Lck	lymphocyte-specific cytoplasmic protein tyrosine kinase
LIME	lck-interacting membrane protein
MAPK	mytogen-activated kinase
NFκB	nuclear factor κB
NTAL	non-T cell activation linker
PBS	phosphate-buffered saline
PEP	PEST domain-enriched tyrosine phosphatase
PI3K	phosphatidylinositol-3 kinase
PLC	phospho lipase C
SDS PAGE	SDS polyacryamid gel electrophoresis
SH2	Src homology domain 2
SHIP	SH2-domain containing inositol phosphatase
SHP-1	Sh2 domain containing PTP
SIT	SH2-domain-containing protein tyrosine phosphatise (SHP2)-interacting transmembrane adaptor protein
SLP-76	SH2 domain containing leucocyte protein of 76 kDa
SOS	son of sevenless homolouge

SP	single positive
Src	Src homology
TBSM	tyrosine based signalling motif
TCR	T cell receptor
Tg	transgenic
TN	triple negative
TRAP	transmembrane adaptor protein
TRIM	TCR interacting transmembrane adaptor protein
VAV	guanine nucleotide exchange factor
Zap70	zeta associated protein of 70 kDa

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Appendix I.

Antibodies used in this study

FACS

Antibody	Fluorophore	Cat.Nr.	Company
CD8	FITC	553031	BD
CD8	PE	553033	BD
CD8	CyCh	553034	BD
CD8	APC	553035	BD
CD4	FITC	553055	BD
CD4	PE	553049	BD
CD4	CyCh	553050	BD
CD4	APC	553051	BD
CD5	FITC	553021	BD
CD5	APC	550035	BD
βTCR	FITC	553171	BD
βTCR	PE	553172	BD
βTCR	CyCh	553173	BD
aCD3	FITC	553062	BD
αCD3	PE	553064	BD
αCD3	CyCh	553065	BD
aCD3	APC	553066	BD
CD44	PE	553134	BD
CD44	CyCh	553135	BD
CD69	PE	553237	BD
CD122	PE	553362	BD
CD62Li	PE	553151	BD
CD25	PE	553866	BD
CD45.1	PE	553776	BD
CD45.2	FITC	553772	BD
Vα2 TCR	FITC	553288	BD
T3.70	FITC	11-99-30-85	Bioscience
T3.70-biotin		11-99-30-82	Bioscience
αBRDU	FITC	36634K	BD
αBRDU-Isotype	FITC		BD
Annexin-V	PE	556421	BD
Bcl-2 (Kit)	FITC	554221	BD
Bcl-2 Isotype (Kit)	FITC	554221	BD

Appendix II

Antibody	Conjugate	Clone	Company
CD3	biotin	145-2C11	BD
CD4	-	GK1.5	BD
CD28	-	37.51	BD
LAT	-	-	Transduction Lab.
Zap70	-		Santa Cruz
P-Tyr	-	4G10	-
p-ERK	-	-	Cell Signalling
p-JNK	-	-	Cell Signalling
p-P38	-	-	Cell Signalling
P38	-	-	Cell Signalling
p-LAT(Y171, Y191)	-	-	Cell Signalling
p-LAT (Y226)	-	-	Upstate
LAT	-	-	Gift from Dr.Weiguo Zhang
p-Zap70 (Y319, Y293)	-	-	Cell Signalling
Zap 70	-	-	Transduction Laboratories

Appendix III

PCR programs and master mixes used for genotyping

program	**SIT**	**master mix 25µl**	
1=94C° 1 min		genomic DNA	1.5ul
2=94C° 40 sec		Primer 1 (50pmol/µl)	0.2µl
3=65C° 1 min		Primer 2 (50pmol/µl)	1.1µl
4=72 C° 1 min		Primer 3 (50pmol/µl)	1.0µl
5=Go to 2 29 times		dNTP (20µM each)	0.25µl
6=72C° 3 min		10x Buffer	2.5µl
7=4C° for ever		Taq polym.	0.2µl
		H_20	18.25µl

program	**HY**	**master mix 25µl**	
1=94C° 3 min		genomic DNA	1.5µl
2=94C° 30 sec		Primer 1 (50pmol/µl)	1.0µl
3=60C° 45 sec		Primer 2 (50pmol/µl)	1.0µl
4=72 C° 45 sec		dNTP (20µM each)	0.25µl
5=Go to 2 25 times		10xBuffer	2.5µl
6=72C° 10 min		Taq polym.	0.2µl
7=4C° for ever		H_20	18.55µl

program	P14	master mix 25µl	
1=94C° 1 min		genomic DNA	1.5ul
2=94C° 30 sec		Primer 1 (50pmol/µl)	0.5µl
3=55C° 30 sec		Primer 2 (50pmol/µl)	0.5µl
4=72 C° 1.30 min		dNTP (20µM each)	0.25µl
5=Go to 2 30 times		10xBuffer	2.5µl
6=4C° for ever		$MgCl_2$	0,225µl
		Taq ploym.	0.2µl
		H_20	20,82 µl

program	OT-I	master mix 25µl	
1=94C° 3 min		genomic DNA	1.5µl
2=94C° 30 sec		Primer 1	0.25µl
3=52C° 30 sec		Primer 2	0.25µl
4=72 C° 30 sec		Primer 3	0.125µl
5=Go to 2 39 times		Primer 4	0.125µl
6=72C° 2 min		10xBuffer	2.5µl
7=4C° for ever		5xEnhancer Q	5.0µl
		Taq polymerase	0.5µl
		H_2O	14.5µl

program	Rag1	master mix 25µl	
1=94C° 3 min		genomic DNA	1.5ul
2=94C° 0.30 min		Primer 1: (50pmol/µl)	0.5µl
3=55C° 1 min		Primer 2: (50pmol/µl)	0.2µl
4=72C° 1 min		Primer 3: (50pmol/µl)	0.2µl
5=Go to 2 35 times		dNTP (20µM each)	0.25µl
6=72C° 2 min		10xBuffer:	2.5µl
7=4C° for ever		Taq polym.	0.13µl
		H_20	17.7µl

program	Fyn	master mix 25µl	
1=94C° 3 min		genomic DNA	1,5µl
2=94C° 30 sec		Primer 1: (50pmol/µl)	0.5µl
3=60C° 45 sec		Primer 2: (50pmol/µl)	0.5µl
4=72C° 45 sec		Primer 3: (50pmol/µl)	0.5µl
5=Go to 2 30 times		Primer 4: (50pmol/µl)	0.5µl
6=72C° 10 min		dNTP (20µM each)	0.25µl
7=4C° for ever		10xBuffer	2.5µl
		DMSO:	1.25µl
		H_20	14.8µl

CURRICULUM VITAE

Personal informations

Name: Vilmos POSEVITZ
Date of birth: 12th December 1973
Place of birth: Budapest, Hungary
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Languages: Hungarian (mother tongue), English (fluent) German (basic)

Education

2003-2007	PhD student Otto-von-Guericke University, Magdeburg, Germany
1998-2003	Biologist: University of Szeged, Szeged, Hungary

Work places:

2007-	Ludwig Institute of Cancer Research, Lausanne, Switzerland
2003-2007	Institute of Molecular and Clinical immunology, Magdeburg, Germany

Publications:

V. Posevitz, B. Arndt, T. Krieger, N. Warnecke, B. Schraven and L. Simeoni 2008 Regulation of T-cell homeostasis by the transmembrane adaptor protein SIT J. Immunol. **180**(3):1634-42

Simeoni, L., V. Posevitz, U. Kolsch, I. Meinert, E. Bruyns, K. Pfeffer, D. Reinhold, and B. Schraven. 2005 The transmembrane adapter protein SIT regulates thymic development and peripheral T-cell functions. Mol.Cell Biol. **25:**7557-7568.

Simeoni, L., M. Smida, V. Posevitz, B. Schraven, and J. A. Lindquist. 2005 Right time, right place: the organization of membrane proximal signaling. Semin Immunol **17:**35-49.

V. Posevitz., C. Vizler, S. Benyhe, E. Duda, and A. Borsodi. 2003 Restraintstress and anti-tumor immune response in mice. Acta Biol. Hung. **54**:167-76.

Grants and Scholaships:

01.2006-03.2007 Promotion for PhD students from German Federal Ministry for Education and Research (BMBF) NBL-3 program.

Scholarship from the University of Szeged, Hungary (1999/2000, 2000/2001, 2001/2002, 2002/2003)

Awards and prizes:

Signal Transduction Socitey (STS) membership in Germany from 2005-

Young Researchers's Competition in 2002, local round, Szeged, Hungary (29th November, 2002): 3rd prize and qualified for the national round (14-17 April, 2003)

Poster:

V. Posevitz , Schraven B, Simeoni L, The transmembrane adaptor protein SIT regulates $CD8^+$ T-cell homeostasis

1st Joint Meeting of European National Societies of Immunology
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September 6-9, 2006 Paris, France