Inhibition of T cell receptor signaling by cholesterol sulfate, a naturally occurring derivative of membrane cholesterol

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Most adaptive immune responses require the activation of specific T cells through the T cell antigen receptor (TCR)–CD3 complex. Here we show that cholesterol sulfate (CS), a naturally occurring analog of cholesterol, inhibits CD3 ITAM phosphorylation, a crucial first step in T cell activation. In biochemical studies, CS disrupted TCR multimers, apparently by displacing cholesterol, which is known to bind TCRβ. Moreover, CS-deficient mice showed heightened sensitivity to a self-antigen, whereas increasing CS content by intrathymic injection inhibited thymic selection, indicating that this molecule is an intrinsic regulator of thymocyte development. These results reveal a regulatory role for CS in TCR signaling and thymic selection, highlighting the importance of the membrane microenvironment in modulating cell surface receptor activation.

The major function of T lymphocytes is to mediate, directly or indirectly, immune responses against foreign antigens while maintaining tolerance to endogenous (self)-antigens. Specificity is achieved through the TCR, which can bind to peptide antigens bound to major histocompatibility complex (MHC) molecules on other cells. The TCR is expressed as a multisubunit membrane receptor that includes the antigen-recognizing heterodimer TCRαβ (or TCRγδ) and a signaling module typically composed of three CD3 dimers: CD3ζζ, CD3εζ, and CD3ζζζ. All eight subunits in the TCR complex are type 1 transmembrane proteins. Engagement of the TCR with specific peptide–MHC ligands initiates a transmembrane signal to trigger the phosphorylation of intracellular immunoreceptor tyrosine-based activation motifs (ITAMs) in CD3 subunits. This phosphorylation then induces a series of signaling events in the T cell, including adaptor protein phosphorylation, formation of signaling complexes, intracellular calcium flux, immunological synapse formation, cytokine secretion and cell proliferation.

Despite the typically weak affinity of the TCR for its peptide–MHC ligand (1–150 nM), a given TCR is highly specific and sensitive for a particular peptide–MHC complex. It has been shown that a single antigenic peptide together with endogenous peptides presented on MHC can trigger cytokine secretion in CD4+ T cells. These paradoxical observations of low affinity and high sensitivity are thought to be at least partially reconciled by the antigen-independent clustering of TCRs on the surface of T cells, reflecting both an intrinsic tendency of TCRs to self-associate (referred to as nanoclustering) and their confinement on specific cytoskeleton-connected structures known as protein islands. Both phenomena result in a higher local concentration of TCRs on the T cell membrane and may be important for T cell sensitivity by enhancing the avidity to multimeric peptide–MHC complexes, enabling cooperativity between TCRs and allowing rare agonist peptide–MHC to rebind quickly to signal continuously.

T cell sensitivity to a specific ligand is also tightly regulated during development. Weak antigenic peptides that are unable to activate mature effector T cells can efficiently induce selection in immature CD4+CD8+ (double-positive (DP)) thymocytes. The resulting thymocyte selection is driven by successful triggering of TCR signal transduction. Moreover, TCR nanoclustering increases from naive T cells to effector and memory T cells, which contributes to the enhanced TCR sensitivity. Thus, effector and memory T cells are more efficient at TCR signaling and more responsive to peptide–MHC than naive T cells. These results highlight that intrinsic regulators of TCR signaling are involved in modulating T cell responsiveness at different developmental stages.

Although several hundred different lipid species exist in cell membranes and ~30% of the eukaryotic genome encodes transmembrane proteins, little is known about the relationship between these membrane lipids and proteins. It has been shown that the transition of the epidermal growth factor receptor from a low-activity monomeric state to a high-activity dimeric state can be inhibited by an interaction with the ganglioside GM3. Importantly, cholesterol specifically associates with the TCRβ chain and is necessary for TCR nanoclustering and high avidity to multivalent peptide–MHC.
Other studies have shown that negatively charged phospholipids are involved in regulation of the TCR–CD3 conformation. Interactions between the CD3ε cytoplasmic domain and phospholipids might decrease the accessibility of phosphorylation sites in ITAMs and also that the rise in intracellular Ca²⁺ that accompanies T cell activation dissociates the CD3ε cytoplasmic domain from the cell membrane to release the ITAM tyrosine residues to facilitate phosphorylation. 

Here we studied the role of CS in modulating TCR signal transduction and its physiological implications on T cell development. We found that TCR signaling is repressed by CS, which is thought to function as a stabilizer of the cell membrane. As a sulfated derivative of membrane cholesterol, CS disrupted cholesterol-driven TCR multimers by replacing cholesterol bound to TCRβ. Moreover, the amount of CS was differentially regulated during thymocyte maturation, suggesting that the CS/cholesterol ratio may have a role in thymic selection. Increasing CS content led to apoptosis in DP cells, seemingly because it depressed the ability of those cells to be positively selected. In contrast, male mice deficient in SULT2B1, the major enzyme responsible for sulfating cholesterol, showed enhanced thymocyte sensitivity to the HY self-ligand in a T cell–autonomous manner. Thus CS has an important role in modulating T cell signaling during thymocyte development.

**RESULTS**

**CS inhibits TCR signaling**

Among the different analogs derived from cholesterol, CS is the most abundant sterol sulfate in human plasma. Previous studies have shown that the relative proportions of CS and cholesterol determine the phase separation and domain curvature in model membranes. To examine the possible influence of CS in T cell reactivity, we incubated SC.C7 T cells (specific for a moth cytochrome C peptide, amino acids 88–103, in I-ß²) isolated from lymph nodes of transgenic mice with CS-supplemented medium and examined CD3ζ phosphorylation upon anti-CD3ζ TCR stimulation. Flow cytometry data showed that CS pretreatment resulted in substantially attenuated anti-CD3-induced CD3ζ phosphorylation (Fig. 1a,b and Supplementary Fig. 1b). Furthermore, in both αβ T cells and γδ T cells, CS treatment blocked TCR-mediated phosphorylation of the S6 ribosomal subunit (Fig. 1c–e), a key downstream component of the TCR signaling pathway that controls protein synthesis. Consistent with the phosphorylation data, TCR-induced upregulation of the activation marker CD69 was also inhibited in CS-treated T cells (Supplementary Fig. 2). We also observed a similar inhibitory effect on S6 phosphorylation in SC.C7 T cell blasts after adenovirus-mediated overexpression of SULT2B1 (ref. 34) (Supplementary Fig. 3a,b). In contrast, CS did not affect phorbol ester (PMA)- and ionomycin-mediated S6 phosphorylation and CD69 upregulation that bypasses TCR stimulation (Fig. 1c and Supplementary Fig. 2). Taken together, these data suggest that CS specifically interacts with the TCR to inhibit transmembrane signaling without interfering with downstream components of the signaling pathway.

We also measured the effect of CS on interleukin 2 (IL-2) secretion induced by a lipid bilayer loaded with a moth cytochrome C peptide (MCC88–103) bound to I-ß² (pMCC–I-ß²) and costimulatory proteins. We found that the secretion of IL-2 was lower in CS-treated SC.C7 T cells than in DMSO-treated cells (Fig. 1f). Finally, we assessed T cell responses to mouse CH27 antigen-presenting cells (APC)-pulsed with MCC peptides. In line with the above data, secretion of IL-2 was reduced in both CS-treated and SULT2B1-overexpressing T cells (Fig. 1g and Supplementary Fig. 3c), indicating that CS also inhibits T cell activation by antigen-pulsed APCs. These results show that CS inhibits T cell activation at a very early stage, where TCR engagement with its peptide–MHC ligand triggers CD3ζ phosphorylation.

**CS decreases TCR avidity**

We then investigated whether CS influences the peptide–MHC binding avidity to the TCR by staining DMSO- or CS-treated SC.C7 T cells with fluorescent pMCC–I-ß² tetramers. Our flow cytometry data showed that increasing CS abundance strongly decreased the binding of tetramers to the T cells (Fig. 2a), suggesting reduced avidity between TCR and multivalent peptide–MHC. Consistent with the tetramer data, the binding of divalent antibodies directed...
against either the variable region or the constant region of TCRβ chain was also reduced by CS (Fig. 2a). In contrast, CS did not affect the binding of monovalent single-chain variable fragment (scFv) to the same epitope of TCRβ chain (Fig. 2a). The observation that both peptide–MHC tetramer and divalent antibody binding were impaired but monovalent antibody binding was not indicated that CS could have an effect on the spatial organization of the TCR on the cell surface.

CS disrupts TCR nanoclusters

It has been suggested that cholesterol has an important role in driving the formation of TCR nanoclusters and that TCR nanoclustering enhances avidity toward peptide–MHC tetramers.11 To investigate the effect of CS on TCR nanoclustering, we isolated native, monomeric TCRs from lysates of M.mCd8−SBP hybridoma T cells (expressing SBP-tagged mouse 2B4 TCRs) using the detergent digitonin7 and reconstituted the TCRs in large unilamellar vesicles (LUVs) with defined lipid components.11 We lysed the proteoliposomes of different lipid composition with 0.5% Brj96 V to maintain any TCR nanoclusters that had formed in the liposomes. We then analyzed the TCR monomers and nanoclusters by Blue native PAGE. We found that high-molecular-weight TCR nanoclusters can form in the proteoliposomes containing the ternary lipid mixture of phosphatidylcholine (PC), cholesterol and sphingomyelin (SM) but not in proteoliposomes containing only PC (Fig. 2b), as reported11. When cholesterol was replaced by CS in the ternary mixture, TCR nanoclusters were not detectable above background (Fig. 2b), demonstrating that CS does not support nanocluster formation. Compared with cholesterol-containing proteoliposomes, fewer low-molecular-weight TCR nanoclusters formed in the proteoliposomes containing both cholesterol and CS together with PC and SM, suggesting that CS can affect TCR nanocluster formation even in the presence of cholesterol (Fig. 2b).

To test whether CS can disrupt existing TCR multimers in living cells, we incubated T cells with DMSO or CS and purified TCRs from the treated T cells to analyze TCR nanoclustering by Blue native PAGE. Consistent with our in vitro proteoliposome data, we found a strong reduction in the TCR nanoclusters extracted from CS-treated cells (Fig. 2c). In contrast, high-molecular-weight complexes of the transferrin receptor (TIR) were not sensitive to CS treatment (Fig. 2d). We conclude that CS can disrupt TCR–CD3 nanoclusters in a manner that is specific to the TCR and that this may be a major mechanism by which CS disrupts signaling.

CS displaces cholesterol from TCRβ

To investigate whether there is an interaction between CS and the TCR, we affinity purified TCR–lipid and TIR–lipid complexes from DMSO- and CS-treated cells, extracted the protein-associated lipids with organic solutions and quantified the CS with mass spectrometry. We calculated the ratio of CS concentration in cells with CS

Figure 2 CS disrupts TCR nanoclusters. (a) Flow cytometry analyzing 5C.C7 T cells treated with DMSO (red) or CS (blue) and stained with pMCC–I-Ek tetramer, anti-Vβ3, anti-Cβ or anti-Cβ scFv. (b) Blue native PAGE and immunoblot analysis of TCR nanoclusters from proteoliposomes with various lipid compositions. The ferritin markers f1 and f2, corresponding to 440 kDa and 880 kDa, are shown. Band intensities of nanoclustered and monomeric TCRs were quantified with the LI-COR Odyssey infrared imager and shown as a ratio of the two forms. Cho, cholesterol. (c,d) Blue native PAGE and immunoblot (IB) analysis of TCR (c) and TIR (d) nanoclusters from cells treated with DMSO or CS. Band intensities of nanoclustered and monomeric TCR or TIR were quantified with the LI-COR Odyssey infrared imager and shown as a ratio of the two forms. **P < 0.01; ***P < 0.001; ****P < 0.0001; NS, not significant, unpaired t-test; error bars, mean and s.e.m. (b,c,d). Data are from one experiment representative of three independent experiments with similar results (a–d) or three independent experiments with n = 3 (b) or 2 (c,d) biological replicates.
supplementation to that in untreated cells. Notably, we found a 200-fold accumulation of CS copurified with the TCR, compared to a <100-fold accumulation of CS copurified with the TIR, as determined by mass spectrometry (Fig. 3a and Supplementary Table 1). These results indicate that CS preferentially associates with the TCR in the cell membrane.

CS is much less abundant than cholesterol under normal physiological conditions. Because CS is able to reduce TCR nanoclusters despite an excess of cholesterol in the plasma membrane, we investigated whether it could compete with cholesterol for TCR binding in vitro using a lipid–protein pulldown method. T cell lysates were incubated with cholesterol-conjugated beads and various free lipids were added to compete with the preformed interaction between cholesterol and TCR. Indeed, less TCR remained on the cholesterol-conjugated beads in the presence of free CS than with free cholesterol, suggesting that CS can disrupt the binding of cholesterol to the TCR–CD3 complex (Fig. 3b). The efficiency with which CS displaced cholesterol was similar to that of digitonin, which can break down TCR nanoclusters into monomers (Supplementary Fig. 4).

We next used photoactivatable cholesterol crosslinking to assess whether CS affects the TCR–TCR interaction in living cells. We cultured T cells with photoactivatable tritiated cholesterol then added CS to compete with the preformed binding of cholesterol and TCR. We used UV light to activate photocholesterol to covalently crosslink it to proteins in close proximity. We then purified TCRs from cellular lysates, separated TCR subunits by SDS–PAGE and analyzed the results by autoradiography and immunoblotting (Fig. 3c). We concluded that cholesterol binds to the TCRβ subunit on the basis of the molecular weight and N-glycosylation, verified by PNGase F treatment (Fig. 3c), consistent with published studies. Cholesterol binding to TCRβ was decreased in the presence of CS (Fig. 3c), indicating that CS can directly disrupt cholesterol binding to TCRβ in living T cells. Taken together, these data show that the TCR–CD3 complex involves CS preferentially compared to the TIR and that CS can efficiently prevent cholesterol binding to the TCR, probably through direct competition.

**Regulation of CS contents in thymocytes**

Because thymic selection involves markedly different sensitivities to ligands as thymocytes mature, we asked whether the abundance of CS is modulated in different developmental stages. We obtained >99% pure thymocyte DP and single-positive (SP) subpopulations by flow cytometry based on CD4 and CD8 staining. We then measured the abundance of mRNAs from Sult2b1 (encoding sulfatase, which metabolizes CS back to cholesterol) by quantitative PCR (Fig. 4a,b). We found that the DP thymocytes had the lowest Sult2b1 expression and the highest Sts expression of the thymocyte subsets (Fig. 4a,b).

This reciprocal gene expression pattern of Sult2b1 and Sts suggested that DP thymocytes may contain low amounts of CS. To test this, we extracted total lipids from sorted DP and CD4+ SP thymocytes and quantified the amounts of CS and cholesterol by mass spectrometry.
In line with the gene expression data, we found that the ratio of CS to cholesterol was threefold lower in DP cells than in CD4+ SP cells (Fig. 4c and Supplementary Table 2).

To assess the impact of CS in thymocyte subsets that actively regulate their CS biosynthesis and metabolism, we incubated CD4+ SP, CD8+ SP and DP thymocytes with various concentrations of CS and monitored TCR signaling by measuring calcium flux (Supplementary Fig. 5). In the absence of CS, TCR-crosslinking antibodies induced calcium flux in all three types of thymocytes (Supplementary Fig. 5). The calcium flux in DP thymocytes was measurably reduced after addition of exogenous CS (5 μM), but SP thymocytes were not affected (Supplementary Fig. 5). Similarly to our signaling data in mature T cells, TCR-mediated calcium flux was impaired by 10 μM CS in all thymocyte populations (Supplementary Fig. 5). These data show that CS abundance, together with other factors, is regulated during thymocyte differentiation, and the lower amount of CS in DP thymocytes may contribute to their enhanced sensitivity.

**CS induces apoptosis in DP thymocytes**

We next investigated the effects of CS modulation on positive thymic selection, in which immature T cells (thymocytes) express TCRs that bind weakly to self-peptide–MHC complexes to mature18,40. At the same time, many T cells that have TCRs specific for self-peptide–MHCs are eliminated through negative selection41. We found that increasing the amount of CS in the thymus by intrathymic injection led to a decrease in the number of total thymocytes (Fig. 5a and Supplementary Fig. 1a). The percentage of DP thymocytes decreased by more than half (from 75% with vehicle to 35% with CS) (Fig. 5b,c).

To test whether the observed reduction of DP cells was due to failure of positive selection, we used annexin V staining to measure the surface exposure of phosphatidylserine (PS), a marker of early apoptosis, as DP thymocytes that fail positive selection die by apoptosis42. CS specifically increased PS exposure on the surface of DP cells but not CD4+ or CD8+ SP cells (Fig. 5d,e), indicating that CS specifically leads to DP cell apoptosis. Moreover, a TUNEL assay showed that numbers of late-stage apoptotic cells in the thymus increased with CS concentration (Fig. 5f,g), confirming the failure of selection in these thymocytes. Intrathymic CS injection did not affect apoptosis in preselection DP thymocytes in OT-I TCR-transgenic Rag2−/−β2m-null mice43,44, suggesting that CS leads to increased DP cell death via prevention of positive selection rather than nonspecific toxicity, although we cannot rule out the possibility that CS impairs the ability of thymic stroma or other non-thymocytes to mediate positive selection (Supplementary Fig. 6). We conclude that the increasing the concentration of CS in the thymus reduces the sensitivity of DP thymocytes to signals from self-peptide–MHC ligands, thus increasing the number of DP cells undergoing apoptosis.

**SULT2B1 deficiency enhances negative selection**

We next investigated the effect of low CS abundance in the thymus by analyzing Sult2b1−/− mice45. The CS/cholesterol ratio in thymocyte lipids from Sult2b1−/− mice was roughly half that of their wild-type counterparts (Supplementary Fig. 7a). The percentages of DP and SP populations in thymocytes and splenocytes were similar between wild-type and Sult2b1−/− mice (Supplementary Fig. 7b).

It has long been thought that self-specific T cells are very efficiently eliminated in the thymus, but recent studies have found that...
some, including HY-specific CD8+ T cells in mice and humans, are not.** HY is a Y-chromosome-encoded antigen commonly used to monitor negative selection in thymocytes.**4,46,47. We measured the frequency of mature HY-specific T cells in the periphery of male and female wild-type and Sult2b1−/− mice (Fig. 6a). We observed a significant reduction (roughly twofold) in the frequency of HY-specific CD8+ T cells in male Sult2b1−/− mice compared to their wild-type counterparts (Fig. 6b,c). The frequency of HY-specific CD8 SP thymocytes was also lower in male Sult2b1−/− mice (**Supplementary Fig. 8**). Furthermore, the intensity of HY tetramer staining was lower in male Sult2b1−/− mice, indicating lower avidity of HY-specific cells (Fig. 6d,e). These data show that Sult2b1−/− mice have heightened sensitivity to negative selection.

To test the possibility that the effect on negative selection described above is mediated by other cell types that thymocytes encounter during maturation, we performed a mixed–bone marrow chimera experiment. Lethally irradiated male B6.SJL mice were reconstituted 1:1 with mixed bone marrow of either wild-type B6.SJL (CD45.2+) and wild-type B6 (CD45.2-) mice or wild-type B6.SJL (CD45.2+) and Sult2b1−/− B6 (CD45.2-) mice. We then examined the HY-specific thymocytes developed from wild-type and Sult2b1−/− bone marrow. Congenic marker gating of the tetramer-positive (tetramer+) populations (Fig. 7a) showed that even in the presence of wild-type cells, fewer CD8+ HY-specific thymocytes developed from Sult2b1−/− bone marrow than from wild-type cells in the same animal (Fig. 7b), although the difference was not as pronounced as in wild-type and Sult2b1−/− mice. These data suggest that the loss of Sult2b1 increases the sensitivity of HY-specific thymocytes to negative selection and that this effect is largely independent of wild-type cells and tissues in that same environment. Thus, the effect of SULT2B1 deficiency is mostly intrinsic to cells carrying that defect.

**DISCUSSION**

Many specialized lipids occur in nature and as components of cell membranes, but their biological roles are largely unknown. A potentially general property may be in modulating the conformation and activity of membrane receptors involved in cell signaling, as seen with the EGF receptor and the GM3 lipid 27 and the TCR–CD3 complex activity of membrane receptors involved in cell signaling, as seen with the EGF receptor and the GM3 lipid 27 and the TCR–CD3 complex 28. Recent studies have found that cholesterol enhances the efficiency of positive selection. Complementarily to thymocyte sensitivity; thus, further lowering it does not increase the efficiency of positive selection.

In conclusion, we present evidence here that CS is a specific, negative regulator of T cell signaling through the TCR–CD3 complex, which is important in regulating the sensitivity of thymocytes during maturation. It may also be involved in other situations involving T cell sensitivity in the periphery or be useful in the treatment of excessive T cell activity. Future work will be needed to fully demonstrate the in vivo relevance of CS. Notably, CS is one of the most abundant sulfated steroid circulating in human plasma, with a concentration of ~6 μM, but high CS concentrations have been observed in certain pathological conditions that include recessive X-linked ichthyosis, cirrhosis of the liver, hypothyroidism and hypercholesterolemia 32,49. It would be interesting to examine T cell function in these conditions. In conclusion, this work adds to increasing evidence that specialized membrane lipids can have important roles as modulators of cell surface receptors involved in cell signaling.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

F.W. and M.M.D. conceived the project. F.W., K.B.-G. and C.Z. worked on TCR proteoliposome reconstitution and BN-PAGE. K.B.-G. and C.Z. performed cholesterol-beads pulldown assay. W.W.A.S. contributed ideas and technical support. F.W. and M.M.D. wrote the manuscript. W.W.A.S. and other authors edited the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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Online Methods

Cell culture and mice. SC.C7 T cells were harvested and cultured as described. Briefly, T cells from lymph nodes of SC.C7 mice were harvested and primed with 10 μM MCC peptides (ANERADLAYLQKQTG). Cells were stimulated on the second day of culture with 50 units/ml of recombinant mouse IL-2. After 7–9 d of culture, T cell blasts were used for in vitro activation and staining assays. Before stimulation, T cell blasts were incubated with 100 μM Cs or DMSO control in RPMI-1640 medium supplemented with 1% lipase-free BSA or 5% lipase-deficient FCS for 2 h at 37 °C. Mouse B lymphoma CH27 cells were preloaded with MCC peptides overnight before stimulation experiments. M.mζ−/− and M.mζ−/−SPB (T cell hybridoma expressing mouse ζ−SPB or human TIR−SPB) cells were cultured as described. Salt25/25− mice were backcrossed onto C57BL/6 genetic background at least 10 times, with genotyping after each generation to choose the breeders with the most C57BL/6 genotype. Final Jackson Laboratory SNP analysis indicated that mice were >99% C57BL/6 background. For the experiment of intrathymic injection, mice were anesthetized, and 20°C57BL/6 background. For the experiment of intrathymic injection, mice were anesthetized, and 20 μM DMSO or Cs (25 mM) was injected into thymus by a Hamilton syringe (10 μl each lobe). For bone marrow–chimera experiments, B6.SJL-Ptprc Pepcβ Boyl (Jackson Laboratory stock number 00214) mice were used. Male wild-type B6.SJL mice were subjected to lethal irradiation (950 rad) then reconstituted with 1:1 mixed bone marrow of wild-type B6.SJL (CD45.2−) and wild-type B6.SJL (CD45.2+) and Salt25/25−/−B6 (CD45.2+) mice. 5 × 10⁶ bone marrow cells were injected into each irradiated recipient intravenously. Ten to twelve weeks after bone marrow transfer, thymocytes were isolated for tetramer staining and flow cytometry analysis. Mice were bred and maintained in the Research Animal Facility at the Stanford University Department of Comparative Medicine Animal Facility in accordance with guidelines of the US National Institutes of Health. Mouse experiments were approved by Stanford University Administrative Panel on Laboratory Animal Care (APLAC).

Flow cytometry. Phospho-flow was used to detect phosphorylation of CD3ζ, CD4, and CD8. Briefly, T cells were stimulated with 10 μg/ml anti-CD3ε for 15 min at 37 °C. Cells were fixed with 1.5% PFA at 25 °C for 10 min. After washes and permeabilization with PBS, T cells were stained for surface markers for 10 min at 25 °C. After washes, T cells were suspended in ice-cold methanol and kept at −30 °C for 30 min to allow permeabilization. After washes with PBS, T cells were stained with fluorescent phospho-specific antibody against p-CD3ζ (BD Biosciences). The data were analyzed with FlowJo software (Treestar) by comparing the ratio of emission of Indo-1 on the Indo-1 Blue (450/50 nm) channel to the Indo-1 Green (525/50 nm) channel.

Tetramer purification. TCR complexes were purified from M.mζ−/−SBP T cells as described. Briefly, M.mζ−/−SBP cells were cultured and lysed with 20 mM Bis-Tris (pH 7.0), 500 μM t-aminocaproic acid, 20 mM NaCl, 2 mM EDTA, 10% glycerol and 1% digitonin or 0.5% Brij 96V as indicated. TCR-tagged TCRs were affinity purified using streptavidin-conjugated agarose (GE Healthcare). Proteins were eluted by 30 min incubation at 4 °C with buffer containing 4 mM free biotin. For quantifying TCR-associated lipids with mass spectrometry, Brij 96V was changed to 0.01% ProteaseMax Surfactant (Promega) during washing steps of the agarose beads. After elution of TCR–lipid complexes with biotin, formic acid was added to the eluates to adjust pH to 2–3. The solution was incubated at 37 °C for 30 min to remove ProteaseMax before lipid extraction.

Proteoliposome reconstitution. Large unilamellar vesicles (LUVs) with different membrane compositions including cholesterol (Sigma), CS (Sigma), soybean phosphatidylcholine and egg sphingomyelin (Lipoid) were prepared as described. Lipid content was determined by thin-layer chromatography. The diameters of the vesicles were measured by dynamic light scattering (Zetamaster S, Malvern Instruments). Approximately 100 ng purified TCR in 100 μl 0.01% Triton X-100 containing buffer was mixed with 100 μl 2 mM prepared liposomes, and 40 μl 0.1% Triton X-100 was added. Samples were agitated for 30 min at 4 °C, and the detergent was removed by adsorption to 2–3 μg of BioBeads SM-2 (BioRad) at 4 °C overnight. To analyze TCR nano-clusters, proteoliposomes were lysed by the detergent-supplemented buffers. Blue native PAGE was performed as described.

Lipid extraction and mass spectrometry quantification. Total lipids of T cells or eluted TCR–lipids complexes were extracted with the two-step Bligh and Dyer method. Briefly, cells were mixed and vortexed T cells with chloroform/methanol (1:2) for 10 min. Then chloroform was added with additional water, and with final vortex. After centrifugation, the lower phase was transferred to a new tube. Chloroform was added to the upper phase to repeat the extraction procedure. Cholesterol-26,26,26,27,27,27-d6 and 5,24-cholesta-3β,7β,12α-triol were added as external internal controls for cholesterol and CS, respectively. After evaporation under nitrogen gas, the lipid extract was dissolved in 200 μl methanol. Cholesterol and CS were separated by Accela 1250 HPLC system (Thermo Fisher Scientific) with a BEH C18 column. The samples were measured using Thermo Fisher Scientific mass spectrometry facility. The MS of cholesterol and CS were operated in APCI and ESI mode, respectively. The experimental details are described in the Supplementary Methods. Cholesterol–TCR interaction assay. The TCR pulldown assay was performed as described. In brief, 20 μl cholesterol-coupled beads and 1 μl T cell lysate containing 1% Brij 96V were incubated for 30 min at 4 °C with rotation. 5.6 μM digitonin, CS, cholesterol or solvent control (DMSO or ethanol) was added to the suspensions to compete the TCR binding of cholesterol beads. The beads were rotated overnight then washed four times with 1 ml wash buffer (200 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1% Brij 96V). Proteins on the beads were separated by 12% SDS–PAGE and subjected to immunoblot analysis.

Photoactive cholesterol binding experiments. The experiments were performed as described. M.mζ−/−SBP cells were incubated for 18 h in lipid-free medium containing the 5,6−3H7-azi-5α-cholestan-3β-ol photocholesterol−mβCD complex (10 μCi/ml). CS was added to the cell suspension at 80 μM concentration for 5 h. Labeled cells were washed and UV irradiated for 8 min. After cell lysis, TCR complexes were affinity purified using streptavidin−conjugated agarose, and PNGase F (New England Biolabs) treatments were performed as indicated. The samples were submitted to SDS–PAGE followed by triton autoradiography and immunoblot.

Tetramer enumeration. Tetramer enrichment was performed similarly as described. Single-cell suspensions were prepared from spleens or thymus harvested from wild-type littermate and Salt25/25−/− mice. Red blood cells (RBCs) were then lysed with ACK buffer. With equal numbers of total cells
from each groups, PE-conjugated HY Uty-WMHHNMDLI:Db tetramers (MBLI) were added to the cell suspension at a 1:10 dilution. Cells were incubated for 1 h at 4 °C and costained with anti-CD8. After washing, cells were resuspended in PBS, 2 mM EDTA and 0.5% BSA, and anti-PE microbeads (Miltenyi Biotec) were added to the cell suspension. Samples were incubated at 4 °C for 15 min and then washed. Cells were applied to prewashed MACS LS columns. After three washes, the bound fraction was eluted and resuspended in FACS buffer (PBS, 2 mM EDTA and 5% FBS). Tetramer-bound fractions were stained with 1:100 dilution of fluorophore-conjugated antibodies to the following markers: F4/80 (BM8), Gr-1 (RB6-8C5), CD11b (M1/70), CD11c (N418), Ter119 (TER-119), CD19 (6D5), CD4 (RM4-5) and LIVE/DEAD Aqua amine (Life Technologies). After staining, the washed samples were collected on a BD LSR II flow cytometer and analyzed using FlowJo (Tree Star).

Statistical analysis. Statistical significance was determined by Student's t-test. Statistical analysis was done with Prism 6 (GraphPad).

50. Swamy, M., Siegers, G.M., Minguet, S., Wollscheid, B. & Schamel, W.W. Blue native polyacrylamide gel electrophoresis (BN-PAGE) for the identification and analysis of multiprotein complexes. Sci. STKE 2006, pl4 (2006).