Themis sets the signal threshold for positive and negative selection in T-cell development

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Development of a self-tolerant T-cell receptor (TCR) repertoire with the potential to recognize the universe of infectious agents depends on proper regulation of TCR signalling. The repertoire is whittled down during T-cell development in the thymus by the ability of quasi-randomly generated TCRs to interact with self-peptides presented by major histocompatibility complex (MHC) proteins. Low-affinity TCR interactions with self-MHC proteins generate weak signals that initiate ‘positive selection’, causing maturation of CD4+ or CD8αβ-expressing ‘single-positive’ thymocytes from CD4+CD8αβ+ ‘double-positive’ precursors. These develop into mature naïve T cells of the secondary lymphoid organs. TCR interaction with high-affinity agonist self-ligands results in ‘negative selection’ by activation-induced apoptosis or ‘agonist selection’ of functionally differentiated self-antigen-experienced T cells5. Here we show that positive selection is enabled by the ability of the T-cell-specific protein Themis4,5 to specifically attenuate TCR signal strength via SHP1 recruitment and activation in response to low- but not high-affinity TCR engagement. Themis acts as an analog-to-digital converter translating graded TCR affinity into clear-cut selection outcome. By dampening mild TCR signals Themis increases the affinity threshold for activation, enabling positive selection of T cells with a naïve phenotype in response to low-affinity self-antigens.

Themis-deficient mice have severely reduced numbers of single-positive thymocytes and peripheral T cells4,5, but the mechanism by which Themis controls T-cell development or function remains obscure. Its rapid phosphorylation after TCR stimulation4,9,10 and subtle signalling defects in Themis−/− double-positive thymocytes suggested a role in proximal TCR signalling4,9,11, although others failed to find any alteration in T CR signalling4,7,10. Such mild or undetected signalling defects seemed incompatible with the strong positive selection defect in Themis-deficient mice. We suspected that activation by antibody-mediated TCR crosslinking may have masked genuine signalling defects that would be revealed with more physiological stimulation. calcium flux is a hallmark of early TCR signalling and is very sensitive to differences in signal strength12. We titrated streptavidin crosslinking for anti-CD3/CD4 antibodies to better mimic graded signal strengths TCRs might generate in vivo. Over a broad concentration range, we found only minor reduction in calcium signal in Themis-deficient thymocytes, consistent with our previous observations4, but of dubious biological relevance (Extended Data Fig. 1). Therefore, we used the well-characterized OT-1 TCR transgenic model13 to enable thymocyte activation by more physiological MHC proteins ligands. Naturally positive selecting and antigen-variant peptides elicit a full spectrum of signal strengths are well documented (Extended Data Fig. 2a). OT-I TCR TCR normally localizes to plasma membrane, which induces p-ERK deeper within the cell12. To determine whether Themis controls the topology of TCR-induced ERK activation, we

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compared p-ERK localization in Themis-deficient or Themis-sufficient pre-selection double-positive thymocytes responding to different ligands (Fig. 2c, d and Extended Data Fig. 7). Weak ligands that normally induce intracellular p-ERK in Themis-expressing cells instead gave a relatively high proportion of membrane-proximal p-ERK in Themis−/− DPs. In aggregate, these data indicate that, without Themis, the ERK cascade

Figure 1 | Differentially regulated Ca<sup>2+</sup> flux in Themis−/− thymocytes. a, b, Themis+/+ or Themis−/− OT-1 pre-selection thymocytes were incubated with indicated stimuli. Results of representative experiment shown in a, same-genotype comparisons in b. c, Summary of Ca<sup>2+</sup> flux differences. Each dot represents one Themis−/− sample normalized to its same-tube Themis+/+ control (sex and age-matched), thus blinding and randomization was unnecessary. Each paired sample represents a biological and technical replicate<sup>30</sup>. Data obtained from eight litters of each genotype over 10 months. Peak-height (top) and area-under-the-curve (AUC, bottom) shown. Statistical significance determined by one-way analysis of variance Dunnnett’s test, with OVA as control comparison. *P < 0.05, **P < 0.01, ***P < 0.001. Minimal sample size needed to obtain P = 0.05, 90% power, estimated based on 1.2-fold difference. a.u., arbitrary units.

d, Ca<sup>2+</sup> flux imaging on supported lipid bilayers. Cells settled on K<sup>+</sup>-monomer or antibody-coated lipid bilayer were imaged before and after adding CaCl<sub>2</sub>. Graphs show mean Fluo-4 intensity (wild type (WT): n = 11, 27, 17, 29; KO: n = 20, 14, 24, 23, for cells stimulated by antibody, OVA, G4 and C tamb, respectively), representative of two experiments. e, Live Ca<sup>2+</sup> imaging of stimulation by RMA-S cells pre-loaded with OVA (n = 5) or G4 (n = 8) peptide, measured as in d.

f, Representative of three experiments.

f, Representative images of OVA-stimulation of cells analysed in e. Themis−/− cells identified by Cy5 staining (red on transmitted light image), Ca<sup>2+</sup> scale on right. g, NFATC2 translocation in thymocytes responding to K<sup>+</sup>-OVA and K<sup>+</sup>−G4 tetramers. Nuclear/cytosolic NFATC2 protein ratios (Extended Data Fig. 4) calculated using CellProfiler, n = 70 cells per data-point. Mean ± s.e.m. shown. P values from unpaired t-test. Representative of two experiments.
was both mis-localized and hyperactivated, so that positive-selecting ligands were mistakenly interpreted as negative/agonist-selectors. Thus, Themis−/− double-positive cells seem unable to precisely distinguish low-affinity from high-affinity TCR signalling.

Both the Ca²⁺ response and ERK signalling cascade occur distal to TCR stimulation. We therefore examined more proximal signalling events to determine where these marked changes in signal transduction to low-affinity ligands were initiated. We found substantially elevated phosphorylation of LAT and PLCγ1 (Fig. 3a), although not SLP-76 (Extended Data Fig. 8), in Themis-deficient versus Themis-sufficient cells upon stimulation with low-affinity ligands. This point of action is consistent with previous reports that Themis interacts with PLCγ1 and is part of the LAT signallingosome.⁴⁻⁷,⁹⁻¹¹. These data demonstrate that Themis−/− cells mount an augmented response to low-affinity ligands, similar to a wild-type response to high-affinity ligands, indicating that Themis restricts TCR signalling in double-positive cells stimulated by low-affinity ligands. SHP1, a cytoplasmic protein tyrosine phosphatase, is an important negative regulator in TCR signalling stimulated by low-affinity ligands. SHP1, a cytoplasmic protein tyrosine phosphatase, is an important negative regulator in TCR signalling.

To test if Themis might limit TCR signalling by controlling SHP1, we analysed SHP1 phosphorylation in Themis−/− and Themis+/+ double-positive cells. Notably, p-SHP1 (but not total protein) was markedly decreased in Themis-deficient cells, clearly opposite to enhanced p-ERK. Indeed p-SHP1 was barely induced in the Themis-deficient cells (Fig. 3b and Extended Data Fig. 9). Moreover, Themis interacted constitutively with SHP1 but p-SHP1 was induced in Themis-SHP1 complexes in response to TCR stimulation (Fig. 3c). Themis–GRB2 binding was constitutive (Fig. 3d)⁴⁻⁷,⁹⁻¹¹. Because activated LCK is a SHP1 substrate,⁸,²², we tested LCK phosphorylation, finding that the activated pY394 form was indeed increased in Themis-deficient thymocytes (Fig. 3e).

These results suggest that Themis ‘caps’ the signal strength by controlling SHP1 activation, moderating strength and kinetics of responses to relatively low-affinity ligands. This allows these self-antigen-experienced cells to mature into naïve T cells. Without Themis, thymocytes receive strong agonist/negative-selection-like signals. To test whether these redirect low-affinity stimulation to induce negative selection, we stimulated pre-selection double-positive thymocytes with Kb-tetramers, and assayed caspase-3 activation, a readout for apoptosis.¹³⁻¹⁶ Indeed, caspase-3 activation was significantly increased in Themis−/− versus Themis+/+ cells in response to ligands that normally induce positive selection, but was similar for ligands that normally induce negative selection (Fig. 4a, left). Reduced activated caspase-3+ cells in OVA-stimulated populations versus weaker ligands is probably due to phagocytosis of dead cells, as OVA signals were clearly stronger based on TCR downmodulation (Fig. 4a, right). Deficiency of the pro-apoptotic protein Bim rescues thymocytes from negative selection.¹¹ Intriguingly, Bim co-disruption rescued the previously reported impaired single-positive thymocyte development in Themis−/− mice and defective CD8SP thymocyte development in OT-I Themis−/− mice (Fig. 4b, c). This indicated that the defect in thymocyte development in Themis−/−

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**Figure 3 | Proximal TCR signalling in Themis-deficient thymocytes responding to positive selecting ligands.** Themis KO or WT pre-selection thymocytes were stimulated with indicated tetramers and analysed by immunoblot. a, LAT and PLCγ1 phosphorylation. b, SHP1 and ERK1/2 phosphorylation. Vav was used as loading control. c, Themis interaction with SHP1 and p-SHP1 (Expt, experiment). d, Themis interaction with GRB2. IB, immunoblot; IP, immunoprecipitation. e, Phosphorylation of LCK in response to different stimuli. The lower molecular weight band in blots probed with anti-p-Y394 is LCK (p56), the higher is FYN (p59). expo, exposure. Representative of 2 (b), 3 (a, c, e) and 6 (d) experiments, respectively.

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Figure 4 | Themis-deficient mice show enhanced negative selection that can be rescued by Bim deficiency, but normal agonist selection. a. Pre-selection thymocytes were stimulated with tetramers for 17 h, then analysed for activated caspase-3.32. Triplicate measurements for two mice per genotype, showing mean ± s.e.m. Right panel shows TCR down-modulation. Representative of 3 experiments. b. Thymocytes were assayed for CD4 and CD8 expression. 4–6-week-old mice of both sexes included, no randomization nor blinding necessary for genetic experiment read by flow cytometry. Sample sizes chosen to obtain P = 0.01, 90% power for fivefold difference. Left, representative plots showing mean ± s.e.m. percentage of each subset labelled. Right, summarized data of absolute numbers for each subset. c. Mice of indicated genotypes as b but expressing OT-1 TCR-transgene, analysed for V32hiCD8+ subset. Summarized data of absolute cell numbers shown. In b and c, each symbol represents a single mouse. Statistics, unpaired t-test (a) and Mann–Whitney test (b, c). Fold increases and P values between indicated samples shown in each scheme. d. Phenotypic analysis of indicated subsets in competitive bone-marrow-reconstituted mice. Data pooled from multiple experiments, shown as mean ± s.e.m. after normalizing Themis+/–/– derived donor cells to Themis+/–/– donor cells (n = 4 pairs of mice per time point except 2 months, 8 pairs). Grey zone indicates ratio between 0.5 and 2. Group sizes based on prior experience.

 Themis, in contrast to generation of peripheral naive T cells, supporting our notion that the role of Themis differs in relation to ligand strength.

This study shows that Themis acts early in the TCR signalling cascade, reducing signal strength in response to low-affinity but not high-affinity MHC proteins ligands. Without Themis, TCR signalling in response to low-affinity MHC proteins ligands mimics normal signalling responses to negative or agonist-selecting ligands, generating stronger signals that redirect the selection outcome. Themis performs this function through controlling recruitment and activation of the phosphatase SHP1, which limits TCR signalling and reportedly affects the thresholds for positive and negative selection19–21, as well as helping to discriminate between agonist and lower-affinity antagonist ligands22. A recent study using a conditional knockout of SHP1, where deletion occurred at the immature double-positive stage of thymocyte development, showed that SHP1 was not required for normal thymocyte development25. It is possible that incomplete deletion of SHP1 mediated by transgenic CD4+Cre may mask the requirement of SHP1 in thymocyte development. Alternatively other phosphatases can act redundantly in selection26. The dominant negative SHP1 transgenes that appeared to show the involvement of SHP1 in development29,30 would likely have blocked other phosphatases in addition to SHP1. We therefore tested whether Themis is able to interact with SHP2. We found that Themis indeed interacts constitutively with SHP2 (Extended Data Fig. 10), indicating that the lack of a Themis+/–/– phenotype in the conditional SHP1 knockout is probably owing to redundancy between phosphatases, raising the possibility that different phosphatases may be important in TCR signalling in different situations.

Themis acts to enforce the threshold between positive and negative/agonist selection, which occurs over a very narrow range of TCR–MHC protein affinities12. Themis causes an analog continuum of TCR affinities to elicit a digital selection outcome—positive versus negative/agonist selection. We speculate that altered Themis expression shifts the selection window, changing the mature TCR repertoire, and therefore affecting disease susceptibility. Indeed, single-nucleotide polymorphisms in the noncoding region between human THEMIS and PTPRK genes have been associated with susceptibility to coeliac disease and multiple sclerosis27–29. More work is needed to understand the connection between these polymorphisms, Themis expression and the aetiology of disease.

METHODS SUMMARY

Animal experiments were performed in accordance with the TSRI Animal Care and Use Committee. Thymocytes from Themis+/+/– or Themis+/– OTI TCR+/2– mice were assayed by flow cytometry. Cells were stimulated with tetramers for flow cytometry or Fluoro4 for imaging, then washed in Ca2+/Mg2+-free medium, 1 mM EGTA. Cells were stimulated with tetramers for flow cytometry, or with antigen-presenting lipid bilayers or peptide-loaded RMA-S cells for imaging. After allowing cells to settle, 1/2 pre-warmed medium containing 2.5 mM CaCl2 and MgCl2 was added (t = 0). For biochemistry, thymocytes were stimulated with tetramers or antibodies, and analysed by western blot as described30–32. Rabbit anti-Themis antibody (Millpore 06-1328) was used in immunoprecipitation and blotting.

Online Content Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Imaging Innovations) was used for the capture as well as for deconvolution and containing 2.5 mM CaCl2 and 2.5 mM MgCl2 was added so that Ca2+ supplemented with 10% (v/v) FCS, 100 U ml−1 penicillin, 10 mg ml−1 streptomycin, 292 mg ml−1 glutamine, 50 mM 2-mercaptoethanol and 25 mM HEPES, pH 7.3 and were incubated for 30 min at 37°C in 5% CO2 with the calcium indicator Indo-1-AM (2 mM; Molecular Probes). Cells were washed twice with cRPMI. For antibody stimulation, cells were stimulated with 10 ng ml−1 of IL-2 for 6 h. Flow cytometry analysis was done on a BD LSR-II digital flow cytometer. Anti-phosphorylated ERK (53-6.7), anti-CD8α (BD Biosciences) antibody (BD Biosciences) was used to block Fc-antibody binding. The following antibodies were purchased from eBioscience: CD4-eF450 (RM4-5), CD5-ef450 (53-7.3), CD69-eF450 (H1.2F3), CD90.2-APC (53-2.1). The following antibodies were purchased from BioLegend: Anti-CD8α-PE (53-5.2), Anti-CD4-eF450 (RM4-5), CD4-FITC (53-7.3), CD11b-APC (A20), CD69-eF450 (1H.2F3), CD90.2-APC (53-2.1). The following antibodies were purchased from Invitrogen: CD8α-PO (5H10). The following antibodies were purchased from Catag: CD4-PO (RM4-5). All antibodies were directly conjugated to fluorophores. An LSR II cell analyser (BD Biosciences) was used for the flow cytometry, and FlowJo software (TreeStar) was used for analysis.

Statistical testing. Statistics were performed as described in Figure legends using Excel, GraphPad Prism, or Igor Pro (WaveMetrics).

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Extended Data Figure 1 | \( \text{Ca}^{2+} \) flux in Themis-deficient thymocytes stimulated by TCR crosslinking. Thymocytes from wild-type or Themis-deficient mice were first stained with saturated amount of anti-CD3/CD4 antibodies and subsequently cross-linked with titrated amount of streptavidin (S.Av). Two independent experiments are shown here.
Extended Data Figure 2 | OT-I TCR system and comparison of Ca\(^{2+}\) flux between Themis-sufficient and Themis-deficient pre-selection thymocytes. 

a, Summary of responses of OT-I T cells and thymocytes to different peptides. Data from references 12, 13 and 37–44. 
b, Representative FACS plots from Fig. 1a are shown here again for illustration (top panel), statistical analysis of Ca\(^{2+}\) flux between Themis-sufficient (+/+) and Themis-deficient (−/−) thymocytes were calculated using Wilcoxon signed rank test (P value listed), each line links cells from a pair of mice being compared in the same tube as described (bottom panel). Results are pooled from multiple experiments as described in Fig. 1 legend.
Extended Data Figure 3 | Comparison of Ca$^{2+}$ flux induced by different methods. Thymocytes from indicated mice were either stimulated with peptide presented on thymocytes themselves (left) or with K$b$-tetramers (right). As shown, similar results were obtained by both stimulation methods.
Extended Data Figure 4 | Quantification of NFATC2 nuclear translocation. Thymocytes were stimulated with ionomycin/PMA (phorbol-12-myristate-13-acetate) (Iono-PMA) to obtain maximal extent of NFATC2 nuclear translocation as a positive control for image analysis. NFATC2 translocation in non-stimulated cells (CONTROL) is used as negative control for image analysis. DAPI and NFATC2 staining are colour-coded as indicated.
Extended Data Figure 5 | Biochemical analyses of ERK phosphorylation in Themis-deficient thymocytes. ERK1 and 2 phosphorylation of indicated thymocytes in response to different stimuli, normalized to VAV. Representative of 4 experiments.
Extended Data Figure 6 | Flow-cytometric analysis of ERK phosphorylation. Thymocytes were prepared and stimulated with Kb-tetramers. Representative FACS plots are shown in a. Data compiled from several experiments. PMA and ionomycin (P+I) treatment was used as a positive control for stimulation to obtain maximal ERK phosphorylation.

b, Same p-ERK data as in Fig. 2b, presented as responses to the different ligands overlaid within the same mouse genotype.
Extended Data Figure 7 | Three-dimensional reconstruction images of negative-selection-like ERK signalling in Themis−/− thymocytes in response to positive-selecting ligands. 

a–c, Themis+/+ or Themis−/− OT-I Tpl−/− pre-selection thymocytes were stimulated with Kb-OVA (a), Kb-G4 (b) or Kb-VSV (c). Localization of p-ERK was determined by specific staining with anti-p-ERK antibody. Nuclei were counterstained with Hoechst 33342, and plasma membrane was labelled with Cy3.5. Top panels represent each separate channel of a single centred plane. Bottom panels represent two different 3D reconstructions of 30 planes (step = 0.2 μm), surface rendering (left) and volume rendering (right). d, Fluorescence line profile analysis of representative cells in a, b and c, green line (p-ERK) blue line (Hoechst) and red line (Cy3.5).
Extended Data Figure 8 | SLP-76 phosphorylation is not affected in Themis-deficient thymocytes. Phosphorylation of SLP-76 was determined in cell lysates. Representative of 2 experiments.
Extended Data Figure 9 | Decreased SHP1 phosphorylation in Themis\(^{-/-}\) double-positive cells. a, Phosphorylation of SHP1 was determined in cell lysates. In this experiment, cell lysates from the same time point after stimulation (0.5, 2 and 5 min, respectively) were grouped together and directly compared on the same gel. b, Quantitation: the intensity ratio of p-SHP1 to total ERK was determined by LiCor Odyssey software.
**Extended Data Figure 10 | THEMIS forms complexes with SHP1 and SHP2.**

HEK293 cells were transiently transfected with the indicated expression vectors. **a, b,** Pull-down assays using Streptactin beads were performed two days after transfection and the precipitate subjected to SDS–PAGE and immunoblotting with anti-haemagglutinin (HA) tag (**a**) and anti-SHP2 (**b**) antibodies, respectively. Representative of 3 (**a**) and 2 (**b**) similar experiments, respectively. Note that HEK293 cells express SHP2 but little SHP1. Cells originally from ATCC, tested negative for mycoplasma within previous 3 months, not short tandem repeat profiled. Constitutive binding of GRB2 to THEMIS has been reported previously.