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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 signaling. 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 proteins (MHCp). Low-affinity TCR interactions with self-MHCp generate weak signals that initiate “positive selection”, causing maturation of CD4 or CD8αβ-expressing “single positive” (SP) thymocytes from $CD4^+CD8\alpha\beta^+$ “double positive” (DP) precursors[1]. 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-cells[2,3]. Here we show that positive selection is enabled by the ability of the T-cell specific protein Themis[4–9] to specifically attenuate TCR signal strength via SHP1 recruitment and activation in response to low but not high-affinity

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Extended data are linked to the online version of the paper.

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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 SP thymocytes and peripheral T-cells[4–8], but the mechanism by which Themis controls T-cell development or function remains obscure. Its rapid phosphorylation after TCR stimulation[4,9,10] and subtle signaling defects in *Themis*$^{-/-}$ DP thymocytes suggested a role in proximal TCR signaling[4,9,11], although others failed to find any alteration in TCR signaling[5–7,10]. Such mild or undetected signaling 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 signaling defects that would be revealed with more physiological stimulation. Calcium flux is a hallmark of early TCR signaling and is very sensitive to differences in signal strength[12]. 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 observations[4], but of dubious biological relevance (Extended Data Fig. 1). Therefore, we used the well-characterized OT-I TCR-transgenic model[13] to enable thymocyte activation by more physiological MHCp ligands. Natural positively-selecting and antigen-variant peptides eliciting a full spectrum of signal strengths are well-documented (Extended Data Fig. 2a). OT-I-*Tap1*$^{-/-}$ mice express low cell-surface MHC-I, so thymocyte development is arrested at the pre-selection DP stage. Therefore OT-I-*Tap1*$^{-/-}$*Themis*$^{-/-}$ or *Themis*$^{+/+}$ thymocytes are an excellent system to study TCR signaling after stimulation with appropriate K^b-tetramers. As expected, Themis-sufficient DP thymocytes gave very low but sustained Ca^{2+} responses to positively-selecting peptides Q4H7, Q7, and G4[12]. Remarkably, Themis-deficient cells responded much stronger than Themis-sufficient cells to these ligands, also giving increased responses to lower-affinity E1 and the natural positive-selector Catnb. Responses to strong agonists A2 and OVA were similar between genotypes (Fig. 1a–c, Extended Data Fig. 2b). Similar results were obtained by stimulation on supported lipid bilayers (Fig. 1d). These results were confirmed by imaging Ca^{2+} in thymocytes recognizing RMA-S cells presenting representative high (OVA) or low (G4) affinity peptides. Themis-deficient thymocytes responded more strongly than Themis-sufficient to G4, but similarly to OVA (Fig. 1e,f; Extended Data Fig. 3). The strong, transient Ca^{2+} response to negative-selecting ligands versus low, sustained signal to positively-selecting ligands[12], are reflected in differential nuclear translocation of NFAT[14]. Themis-sufficient thymocytes induced strong, transient NFAT translocation in response to K^b-OVA tetramer, and delayed but more sustained translocation in response to K^b-G4 (Fig. 1g, Extended Data Fig. 4). In contrast, in Themis-deficient thymocytes, both K^b-OVA and K^b-G4 tetramers induced nearly identical NFAT translocation kinetics, similar to the K^b-OVA response of wild-type thymocytes. These data demonstrate that the role of Themis in thymocyte signaling is manifest in the response to weak ligands, but masked in responses to strong (agonist) ligands. These differences were widest with ligands below the previously-

described positive-negative selection border[12]. Differences were noticeable for ligands slightly above this threshold (weak agonists), but were completely masked by antibody cross-linking.

Appropriate ERK signaling kinetics are critical for positive selection[15–17]. As shown previously, antibody-crosslinking induced slightly weaker ERK phosphorylation in Themis-deficient compared to -sufficient cells[4] (Extended Data Fig. 5). Further analysis of phospho-(p)-ERK by flow cytometry showed roughly equal p-ERK responses induced with K^b-OVA tetramer in both genotypes. However, ERK signaling was faster and stronger in Themis-deficient DP in response to ligands weaker than K^b-OVA (Fig. 2a,b; Extended Data Fig. 6), and confirmed by immunoblot (Extended Data Fig. 5). These data further support the notion that *Themis*$^{-/-}$ cells receive signals induced by classical positive-selecting ligands as activating signals, similar to negative/agonist ligand-induced signals.

The subcellular localization of p-ERK in thymocytes differs with ligand affinity, with negative/agonist selecting stimuli inducing p-ERK more proximal to the plasma membrane than positive selection stimuli, which induced p-ERK deeper within the cell[12]. To determine if Themis controls the topology of TCR-induced ERK activation, we compared p-ERK localization in Themis-deficient or -sufficient pre-selection$^-$ DP thymocytes responding to different ligands (Fig. 2c,d; 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 suggest that, without Themis, the ERK cascade was both mis-localized and hyperactivated, so that positive-selecting ligands were mistakenly interpreted as negative/agonist-selectors. Thus, *Themis*$^{-/-}$ DP cells appear unable to precisely distinguish low from high-affinity TCR signaling.

Both the Ca^{2+} response and ERK signaling cascade occur distal to TCR stimulation. We therefore examined more proximal signaling events to determine where these dramatic changes in signal transduction to low-affinity ligands were initiated. We found substantially elevated phosphorylation of LAT and PLCγ1 (Fig. 3a), though not SLP-76 (Extended Data Fig. 8) in Themis-deficient versus –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 signalosome[4,9,11]. These data demonstrate that *Themis*$^{-/-}$ cells mount an augmented response to low-affinity ligands, similar to a wild-type response to high-affinity ligands, suggesting that Themis restricts TCR signaling in DP cells stimulated by low-affinity ligands. SHP1, a cytoplasmic protein tyrosine phosphatase, is an important negative regulator in TCR signaling (reviewed in ref.18). It is activated by tyrosine phosphorylation, and reported to control the thresholds for positive and negative selection[18–21]. To test if Themis might limit TCR signaling by controlling SHP1, we analyzed SHP1 phosphorylation in *Themis*$^{+/+}$ and *Themis*$^{-/-}$ DP cells. Remarkably, p-SHP1 (but not total protein) was decreased dramatically in Themis-deficient cells, clearly opposite to enhanced p-ERK. Indeed p-SHP1 was barely induced in the Themis-deficient cells (Fig. 3b, 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)[5–7,9]. Because activated LCK is a SHP1

substrate[18,22], 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 DP thymocytes with K^b-tetramers, and assayed caspase-3 activation, a readout for apoptosis[23]. 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 likely 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[24]. Intriguingly, *Bim* co-disruption rescued the previously reported[4–8] impaired SP 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*$^{-/-}$ mice is caused at least partially by misplaced negative selection (agonist selection would not be affected by Bim-deficiency). This suggested the possibility that, while conventional naïve T-cell development requiring weak sustained signaling is severely impaired, agonist-selection of non-conventional T-cells by intermediate affinity ligands might be spared or compensated in Themis-deficient mice. To test this, we simultaneously examined development of wild-type and Themis-deficient T-cells in mixed bone marrow chimeras, avoiding potential homeostatic proliferation artifacts (Fig. 4d). *Themis*$^{+/+}$ vastly outnumbered *Themis*$^{-/-}$ cells amongst conventionally-selected naïve peripheral T-cells, but TCR$\gamma\delta^+$ IELs, TCR$\alpha\beta^+$ CD8$\alpha\alpha$ IELs and liver iNKT-cells, which all are selected from DP by strong TCR signals[2,3,25], showed roughly equal reconstitution by both genotypes. Thus, development of agonist-selected T-cells is not, or at most very mildly, affected by the absence of Themis, in contrast to generation of peripheral naïve 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 signaling cascade, reducing signal strength in response to low-affinity but not high-affinity MHCp ligands. Without Themis, TCR signaling in response to low-affinity MHCp ligands mimics normal signaling 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 signaling and reportedly affects the thresholds for positive and negative selection[19–21], as well as helping discriminate between agonist and lower-affinity antagonist ligands[22]. A recent study using a conditional knockout of SHP1, where deletion occurred at the immature DP stage of thymocyte development, showed that SHP1 was not required for normal thymocyte development[26]. 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

selection[26]. The dominant negative SHP1 transgenes that appeared to show SHP1's involvement in development[19,20] 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*$^{-/-}$-like phenotype in the conditional SHP1 knockout is likely due to redundancy between phosphatases, raising the possibility that different phosphatases may be important in TCR signaling in different situations.

Themis acts to enforce the threshold between positive and negative/agonist selection, which occurs over a very narrow range of TCR-MHCp affinities[12]. 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 celiac disease and multiple sclerosis[27–29]. More work is needed to understand the connection between these polymorphisms, Themis expression, and the etiology of disease.

METHODS SUMMARY

Animal experiments were performed in accordance with the TSRI Animal Care and Use Committee. Thymocytes from *Themis*$^{+/+}$ or *Themis*$^{-/-}$ OT-I-*Tap1*$^{-/-}$ 4–6 week old mice, both sexes, were used. For *ex vivo* experiments, thymocytes were rested 3hr at 37°C before assay. For Ca^{2+} assays, knockout cells were pre-labeled with Cy5, mixed with wild-type cells, loaded with Ca^{2+}-sensitive dye Fluo-4, then washed in Ca^{2+}/Mg^{2+} free medium, 1mM EGTA[30]. Cells were stimulated with tetramers for flow cytometry[30], or with antigen-presenting lipid bilayers or peptide-loaded RMA-S cells for imaging: after allowing cells to settle, 1v/v pre-warmed medium containing 2.5mM $CaCl_2$ and $MgCl_2$ was added (t=0). For biochemistry, thymocytes were stimulated with tetramers or antibodies, and analyzed by western blot as described[4,12]. Rabbit anti-Themis antibody (Millipore #06-1328) was used in immunoprecipitation and blotting.

METHODS

Mice

Themis$^{-/-}$ mice (B6.129S-*Themis*^{tm1Gasc}) were produced at TSRI and are available from Jackson Laboratory (Stock# 010919). C57BL/6 (Thy1.2^{+}CD45.2^{+}) mice were bred at TSRI. OT-I and OT-I-*Tap1*$^{-/-}$ animals were obtained from S. Jameson and K. Hogquist (University of Minnesota, Minneapolis, MN) and Bim knockout mice from L. Sherman (TSRI). All experiments were performed in accordance with the guidelines of the Animal Care and Use Committee of TSRI.

Antibodies

Anti-CD3 (145-2C11), anti-CD4 (RM4.4, RM4.5 and GK1.5), anti-CD8α (53-6.7), anti-CD8β, and anti-Vα2 (B20.1) were from eBioscience or Biolegend. Antibodies against phosphorylated extracellular signal-regulated kinase 1 and 2 (ERK1/2) (T^{202}/Y^{204}, Cat#

9101), phosphorylated PLC-γ1 (Cat# 2821), phosphorylated LAT (Cat# 3584), phosphorylated SHP1 (Cat# 8849), VAV (Cat# 2502), and NFAT1 (Cat# 5861) were from Cell Signaling Technology. Anti-phosphorylated tyrosine (4G10, Cat# 05-1050) and rabbit anti-Themis antibody were from Millipore (Cat# 06-1328). Anti-SHP1 antibody was from Santa Cruz Biotechnology (sc-287). Phospho-Src family (Tyr416) antibody (Cell signaling Cat#2101) cross-reacts with other Src-family kinases phosphorylated at the activating site. As a result probing thymocyte lysates with this mAb shows phosphorylated forms of both p56 Lck and p59 Fyn.

Ca^{2+} flux

Ca^{2+} flux measurement by flow cytometry was performed as previously described[30]. Briefly, thymocyte suspensions were prepared and put in separate tubes. One of the two cell types was loaded for 5 min at room temperature with indodicarbocyanine (Cy5; 1 mg/ml) or was mock treated, followed by washing. Equal numbers of Cy5-labeled cell populations were mixed with the unlabeled sample; for example, OT-I-*Tap1*$^{-/-}$*Themis*$^{+/+}$ (mock) plus OT-I-*Tap1*$^{-/-}$*Themis*$^{-/-}$ (Cy5). Cells were suspended at a density of 2–6 × 10^6 cells per ml in cRPMI (RPMI medium supplemented with 10% (vol/vol) FCS, 100 U/ml of penicillin, 10 mg/ml of streptomycin, 292 mg/ml of glutamine, 50 mM 2-mercaptoethanol and 25 mM HEPES, pH 7.3) and were incubated for 30 min at 37°C in 5% CO_2 with the calcium indicator Indo-1-AM (2 mM; Molecular Probes). Cells were washed twice with cRPMI. For antibody stimulation, cells were stained for 20 min on ice with biotin-conjugated anti-CD3 and anti-CD4 (RM4.4), as well as PerCP-Cy5.5–conjugated anti-CD8α and PE–conjugated anti-CD4 (GK1.5) in cRPPM. Cells were washed once with cRPMI and once with cHBSS (Ca^{2+}-free and Mg^{2+}-free Hank's balanced-salt solution supplemented with 1% (vol/vol) FCS, 1 mM $MgCl_2$, 1 mM EGTA and 10 mM HEPES, pH 7.3) and were resuspended in cHBSS. Cells were prewarmed to 37°C before analysis and were kept at 37°C during event collection on an LSR II (Becton-Dickinson). For cell stimulation, streptavidin (10 mg/ml; Jackson ImmunoResearch) was added to crosslink biotinylated antibodies; alternatively, cells were stimulated with tetramers (NIH tetramer core facility). $CaCl_2$ (2mM) was added during analysis. Mean fluorescence ratio was calculated with FlowJo (TreeStar).

Imaging of Ca^{2+} flux

Knockout cells were pre-labeled with Cy5 as above[30] then both wild-type and knockout cells were mixed and loaded with 2μM Ca^{2+}-sensitive dye Fluo-4. After washing in Ca^{2+}/Mg^{2+} free medium with 1mM EGTA, cells were mixed and added to the 37°C imaging chamber containing either the antigen-presenting lipid bilayer or peptide-loaded RMA-S cells. After allowing cells to settle and starting imaging, 1 volume of pre-warmed medium containing 2.5mM $CaCl_2$ and 2.5mM $MgCl_2$ was added so that Ca^{2+} influx started at t=0.

p-ERK analysis

For intracellular staining of phosphorylated ERK, stimulated cells were fixed with 4% paraformaldehyde for 12 min at room temperature and then plated in microtiter tubes (for phospho-flow analysis) or in poly-L-Lysine coated LabTek II chambers (for imaging). Cells were permeabilized with 0.3% Triton X-100 for 3 min at room temperature. Cells were then

blocked with 5% normal goat serum and incubated with rabbit monoclonal antibody against phospho-ERK, after which they were incubated with Alexa Fluor 488–conjugated Fab fragments against rabbit antibodies (Molecular Probes) for secondary detection (either phospho-flow or microscopy). For flow cytometry analysis, one of the two cell types were pre-labeled with dye Cy5 and the other one was mock labeled. Then the two cell types were mixed in 1:1 ratio and processed for stimulation and staining. Phospho-flow analysis of p-ERK was done on a BD LSR-II digital flow cytometer and the two cell types were distinguished by Cy5 labeling. p-ERK imaging was done as described previously with modification[31]. Briefly, after intracellular staining of p-ERK, nuclei were counterstained with the DNA binding dye 4',6-diamidino-2-phenylindole (DAPI, Invitrogen). All images were captured with a Zeiss Axiovert 200 M inverted microscope. SlideBook software (Intelligent Imaging Innovations) was used for the capture as well as for deconvolution and image analysis, and ImageJ was used for image presentation.

NFAT1 imaging

Nuclear translocation of NFAT1 was imaged as described above regarding p-ERK imaging[31], and analyzed using CellProfiler software (Broad Institute).

Immunoprecipitation and western blotting analysis

Briefly, cells were stained with antibodies against CD3 and CD4 or tetramers on ice for 15 min and stimulated by rapidly warming to 37°C with pre-warmed PBS for indicated times. Cells were then lysed and processed for immunoprecipitation or immunoblot analysis as described in detail in Ref.32. In some cases, after primary antibody incubation, membranes were incubated with goat anti-rabbit IgG (H+L), DyLight 800 conjugated (Pierce, Cat#35571) or goat anti-mouse IgG (H+L), DyLight 680 conjugated (Pierce, Cat#35571), and detected and quantified with LiCor Odyssey infrared imaging system. Human embryonic kidney epithelial cells (HEK293), which express SHP2 but little SHP1[33,34], were transfected by calcium phosphate precipitation and lysates prepared as described[11]. Streptactin-Sepharose beads (IBA BioTAGnology) were used for THEMIS-Strep pull-downs, after which beads were washed three times with lysis buffer. Bound proteins were eluted with 5 mM biotin and subjected to SDS-PAGE[11].

In vitro apoptosis assay

Experiments were performed as described previously[23]. Briefly, pre-selection thymocytes were stimulated with the various tetramers for 17 hours. Apoptosis was determined by staining active Caspase-3 with FITC-conjugated specific antibody following manufacturer's protocol (APO ACTIVE 3 detection kit, Cell Technology Inc, Cat# FAB200-1), and analyzed by FACS.

Phenotyping of agonist-selected T-cells

Agonist-selected T-cells were analyzed in bone marrow reconstituted mice. Briefly, *Themis*$^{+/+}$ (CD45.1^{+}/CD45.2^{+}) and *Themis*$^{-/-}$ bone marrow cells (CD45.2^{+}) were mixed 1:1 and injected into lethally irradiated (2 × 5.5 Gy, separated by 3–4 hrs) B6 recipient mice (CD45.1^{+}). At various times post bone marrow reconstitution, tissues and organs of recipient

mice were harvested and relevant T-cell subsets were analyzed. Splenocytes, IELs and liver mononuclear cells were prepared as previously described[35,36]. Cells were suspended in PBS + 5% FCS + 0.16% azide and anti-CD16/CD32 (2.4G2) Fc-receptor antibody (BD Biosciences, San Diego, CA, USA) was used to block Fc-antibody binding. The following antibodies were purchased from BD Biosciences (San Diego, CA, USA): TCRγδ-FITC (GL3), TCRβ- APC-ef780 (H57-597), NK1.1-FITC (PK136), CD8α-PE (53-6.7), CD8β-FITC (53-5.8), CD19-APC (1D3), CD45.2-PercP-Cy5.5 (104). The following antibodies were purchased from eBioscience (San Diego, CA, USA): CD4-eF450 (RM4-5), CD5-ef450 (53-7.3), CD45.1-PE-Cy7 (A20), CD69-eF450 (H1.2F3), CD90.2-APC (53-2.1). The following antibodies were purchased from Invitrogen CD8α-PO (5H10). The following antibodies were purchased from Caltag: CD4-PO (RM4-5). All antibodies were directly conjugated to fluorophores. An LSR II cell analyzer (BD Biosciences, San Jose, CA, USA) was used for the flow cytometry, and FlowJo software (TreeStar, Ashland, OR, USA) was used for analysis.

Statistical testing

Statistics were performed as described in Figure legends using Excel, GraphPad Prism, or Igor Pro (Wavemetrics, Inc).

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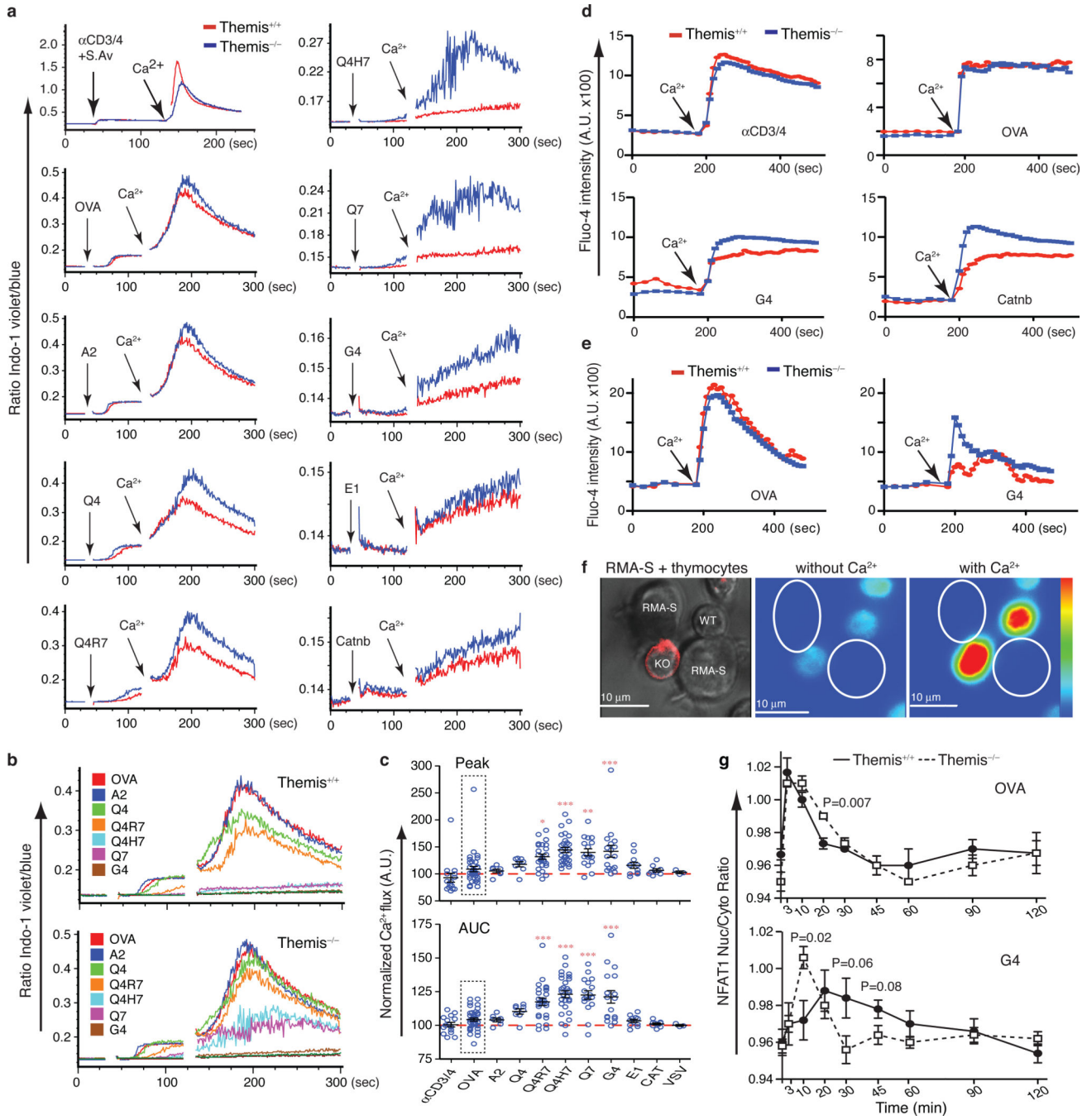


Figure 1. Differentially regulated Ca^{2+} flux in *Themis*$^{-/-}$ thymocytes

(a,b) *Themis*$^{+/+}$ or *Themis*$^{-/-}$ OT-I pre-selection thymocytes were incubated with indicated stimuli. Results of representative experiment shown in (a), same-genotype comparisons in (b). **(c)** Summary of Ca^{2+} flux differences. Each dot represents one *Themis*$^{-/-}$ sample normalized to its same-tube *Themis*$^{+/+}$ control[30] (sex and age-matched), thus blinding and randomization was unnecessary. Each paired sample represents a biological and technical replicate[30]. Data obtained from 8 litters of each genotype over 10 months. Peak-height (upper) and area-under-the-curve (AUC, lower) shown. Statistical significance determined

by 1-way ANOVA Dunnett's Test, with OVA as comparison control. *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. **(d)** Ca^{2+} flux imaging on supported lipid bilayers: cells settled on K^b-monomer or antibody-coated lipid bilayer were imaged before and after adding $CaCl_2$. Graphs show mean Fluo-4 intensity (WT: n=11, 27, 17, 29; KO: n=20, 14, 24, 23, for cells stimulated by Ab, OVA, G4 and Catnb, respectively), representative of two experiments. **(e)** Live Ca^{2+} imaging of stimulation by RMA-S cells pre-loaded with OVA (n=5) or G4 (n=8) peptide, measured as in (d). Representative of three experiments. **(f)** Representative images of OVA-stimulation of cells analyzed in (e). *Themis*$^{-/-}$ cells identified by Cy5 staining (red on transmitted light image), Ca^{2+} scale on right. **(g)** NFAT1 translocation in thymocytes responding to K^b-OVA and K^b-G4 tetramers. Nuclear:cytosolic NFAT1 protein ratios (Extended Data Fig. 4) calculated using CellProfiler, n=70 cells per data-point. Mean±s.e.m. shown. *P<0.05, unpaired t-test. Representative of two experiments.

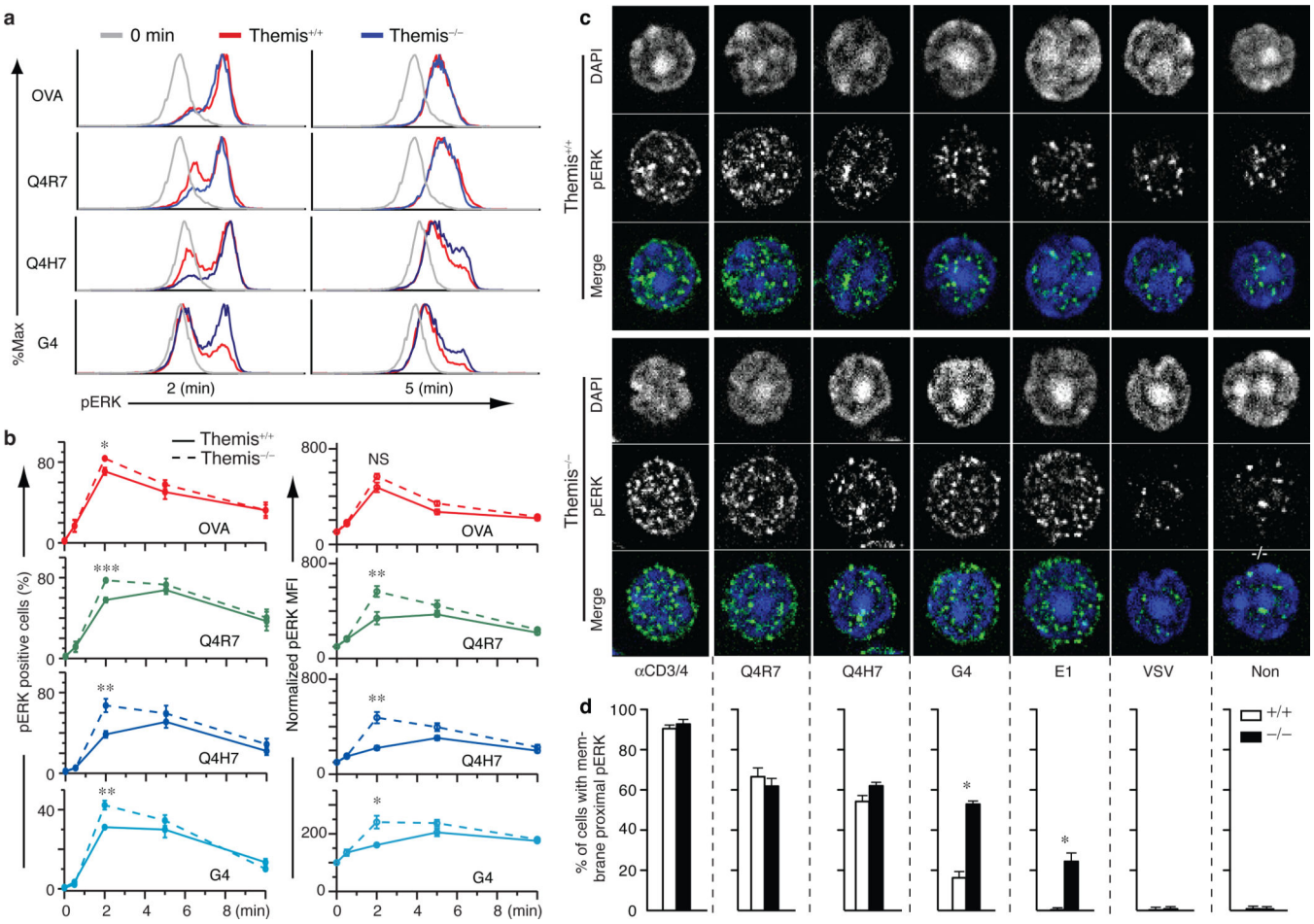


Figure 2. Themis-deficiency allows low-affinity ligands to elicit negative selection-like characteristics of ERK activation

Themis-sufficient or deficient pre-selection thymocytes were stimulated with K^b-tetramers. **(a,b)** ERK phosphorylation detected by flow cytometry following intracellular staining. Representative FACS plots shown in (a) and Extended Data Fig. 6. **(b)** Summarized data compiled from multiple experiments (n=5 for OVA, Q4R7, Q4H7, n=8 for G4), presented as mean±s.e.m. of percentage p-ERK^+ cells (left), MFI (right). Sample sizes estimated as Fig. 1. **P*<0.05, ***P*<0.01, and ****P*<0.001, paired t-test. **(c,d)** Localization of p-ERK determined by staining stimulated thymocytes (2 min) with DAPI and anti-p-ERK[12]. (c) Representative images, (d) Percentage of cells with membrane-proximal p-ERK, n=20 for each condition, mean±s.e.m. Representative of four experiments for each tetramer, two different operators, not blinded. Unpaired t-test on two identical experiments, **P*<0.05.

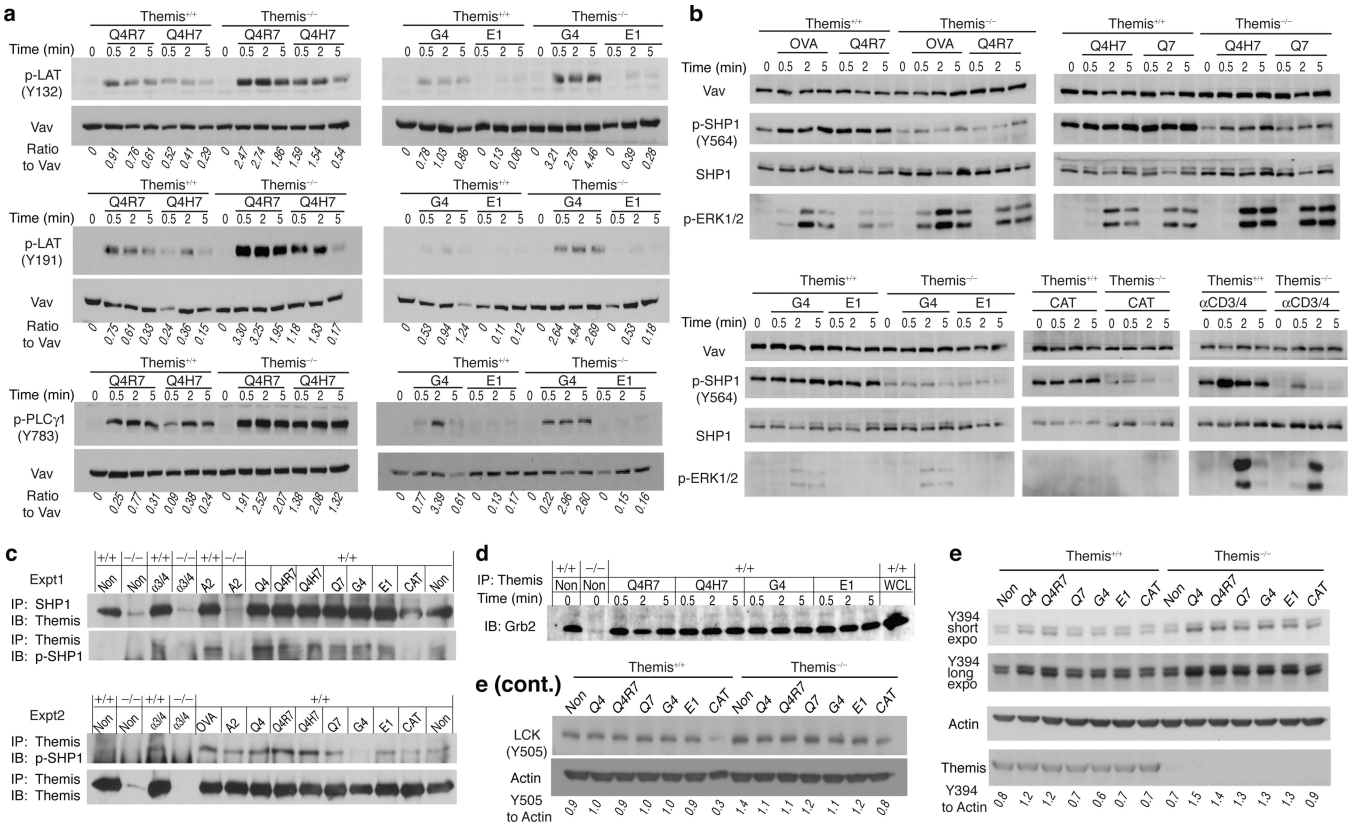


Figure 3. Proximal TCR signaling in Themis-deficient thymocytes responding to positive selecting ligands

Themis KO or WT pre-selection thymocytes were stimulated with indicated tetramers and analyzed by immunoblot. (**a**) LAT and PLCγ1 phosphorylation, (**b**) SHP1 and ERK1/2 phosphorylation, (**c**) Themis interaction with SHP1 and p-SHP1, (**d**) Themis interaction with GRB2. (**e**) Phosphorylation of LCK in response to different stimuli. The lower-molecular mass band in blots probed with anti-p-Y394 is LCK (p56), the higher is FYN (p59). Representative of 2 (b), 3 (a, c, e), and 6 (d) experiments respectively.

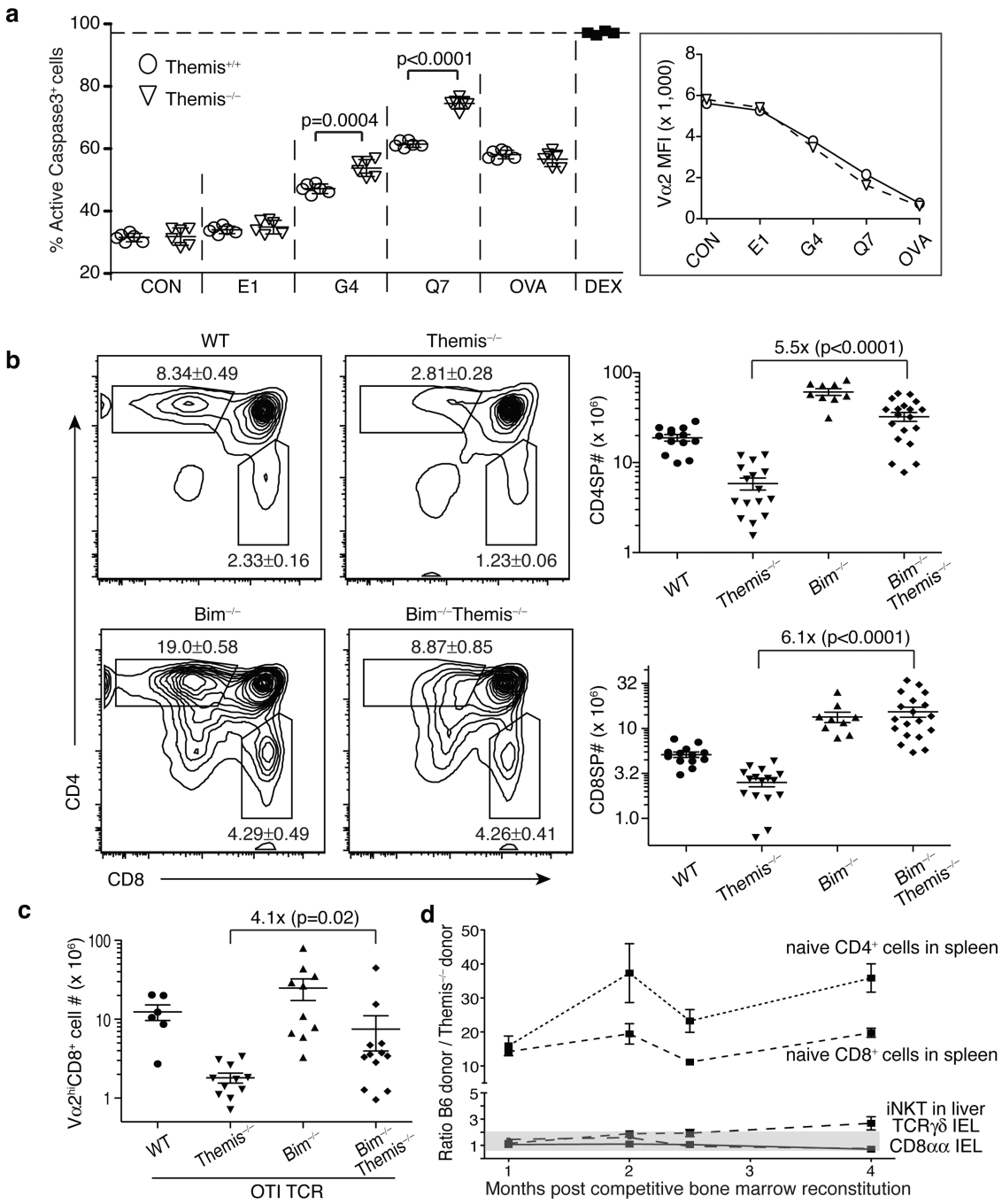
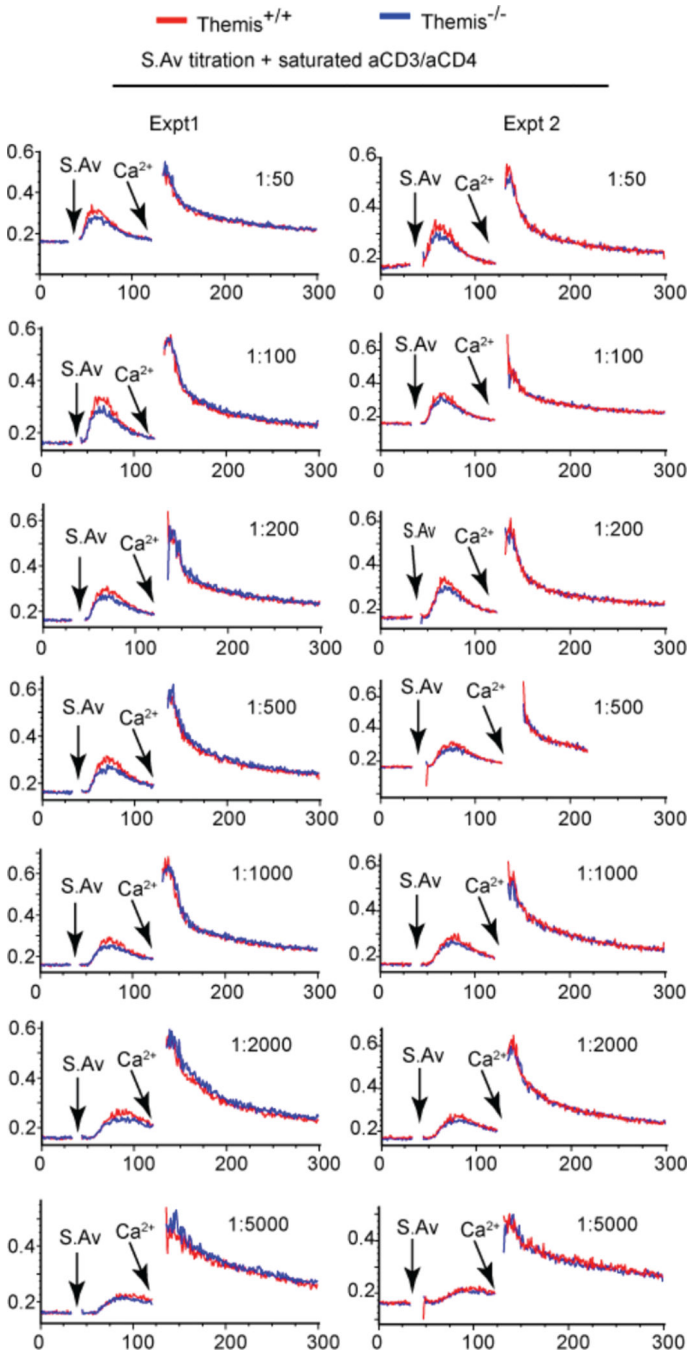


Figure 4. Themis-deficient mice show enhanced negative selection that can be rescued by Bim-deficiency, but normal agonist-selection

(**a**) Pre-selection thymocytes were stimulated with tetramers for 17h, then analyzed for activated caspase-3[23]. Triplicate measurements for two mice per genotype, showing mean ±s.e.m. Right panel shows TCR downmodulation. Representative of 3 experiments. (**b**) Thymocytes were analyzed for CD4 and CD8 expression. 4–6 week-old mice of both sex included, no randomization nor blinding necessary for genetic experiment read by flow cytometry. Sample sizes chosen to obtain *P*=0.01, 90% power for 5-fold difference. Left:

representative plots showing mean±s.e.m percentage of each subset labeled. Right: summarized data of absolute numbers (#) for each subset. (**c**) Mice of indicated genotypes as (b) but expressing OT-I TCR-transgene, analyzed for $V\alpha2^{hi}CD8^{+}$ 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 mice per timepoint except 2 months, 8 pairs). Grey zone indicates ratio between 0.5 and 2. Group sizes based on prior experience.



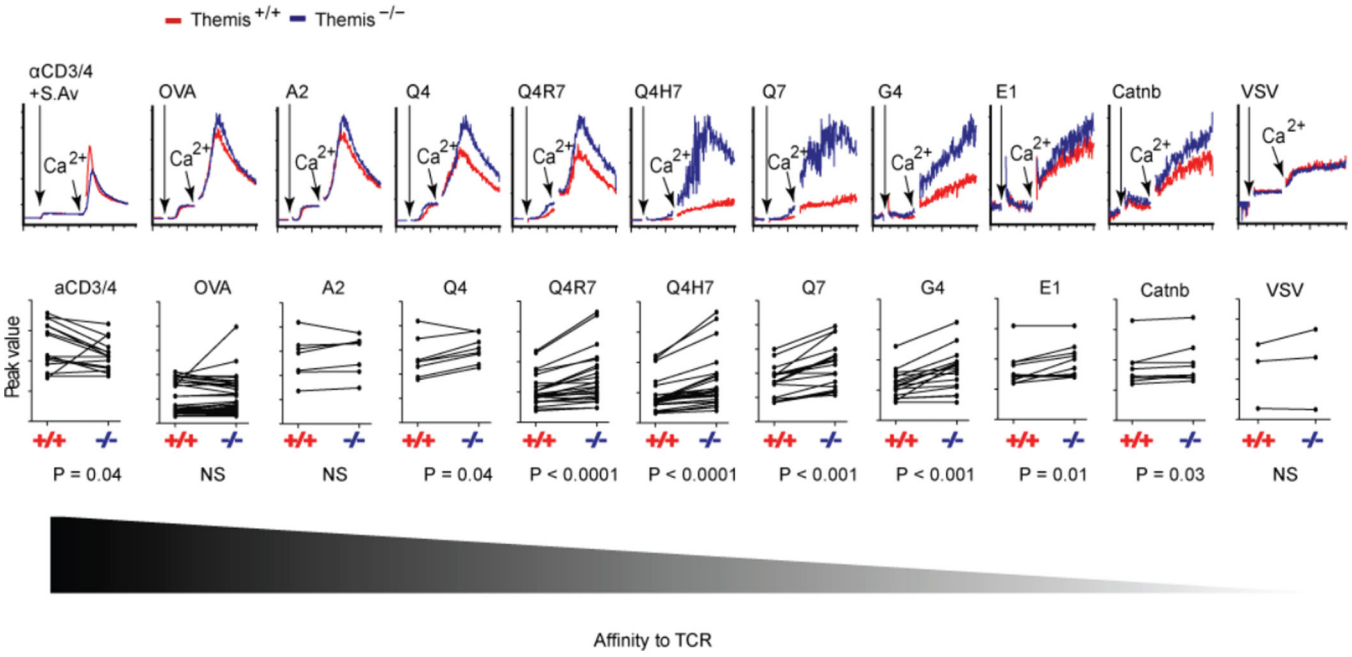
Extended Data Figure 1.

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.

a

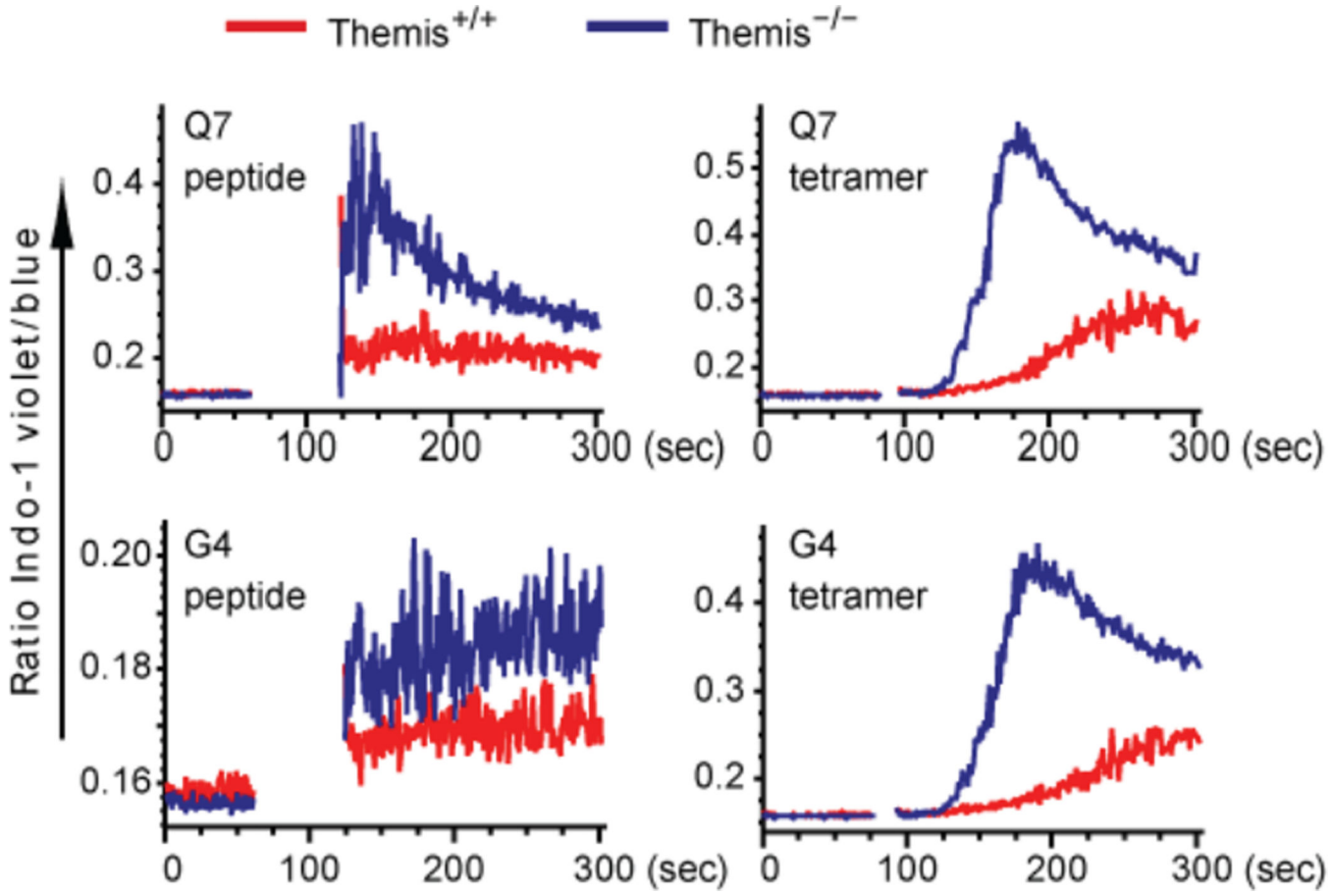
Peptide	Sequence	Activation of mature T cells	FTOC selection (OT-I β2m$^{-/-}$ or *Tap1*$^{-/-}$)	Relative potency (CD69 upregulation)	A_cK_a (2D affinity) (μm^4)	K_d (3D affinity) (μM)	Tetramer avidity (nM)
OVA	SIINFEKL	Strong agonist	Negative selection	1	1 x 10^{-3}	5.9	4
A2	SAINFEKL	Strong agonist	Agonist/negative selection		7 x 10^{-4}	4.4	
Q4	SIIQFEKL	agonist	Agonist/negative selection	39			29
Q4R7	SIIQFERL	agonist	Agonist/negative selection	81			48
Q4H7	SIIQFEHL	Agonist/antagonist	Positive selection	167			51
Q7	SIINFEQL	Agonist/antagonist	Positive selection	268			
G4	SIIGFEKL	Agonist/antagonist	Positive selection	7,515	2 x 10^{-5}	10.0	
E1	EIINFEKL	Antagonist	Positive selection	56,524	5 x 10^{-6}	22.6	
Catnb	RTYTYEKL	Not measurable	Positive selection			136 (10°C)	
Cappa1	ISFKFDHL	Not measurable	Very weak pos. sel^n			211 (10°C)	
P815 or VSV	HIYEFPQL RGYVYQGL	Not measurable	Not measurable	∞	< 10^{-8}	not measurable	
Refs.			15, 16, 37-40	15	41	40, 42-44	15

b



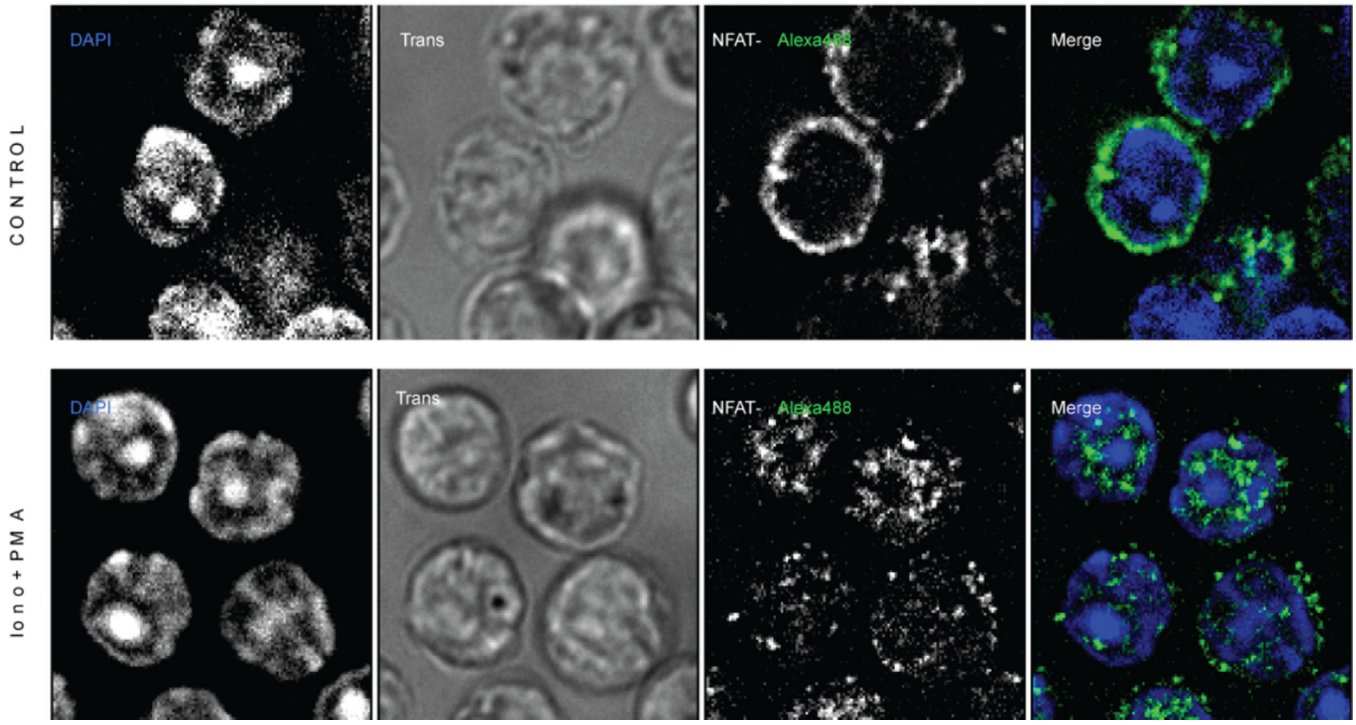
Extended Data Figure 2.

OT-I TCR system and Comparison of Ca^{2+} flux between Themis-sufficient and –deficient pre-selection thymocytes. (a) Summary of responses of OT-I T cells and thymocytes to different peptides. Data from references cited in table. (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 (Ref. 30) (bottom panel). Results are pooled from multiple experiments.



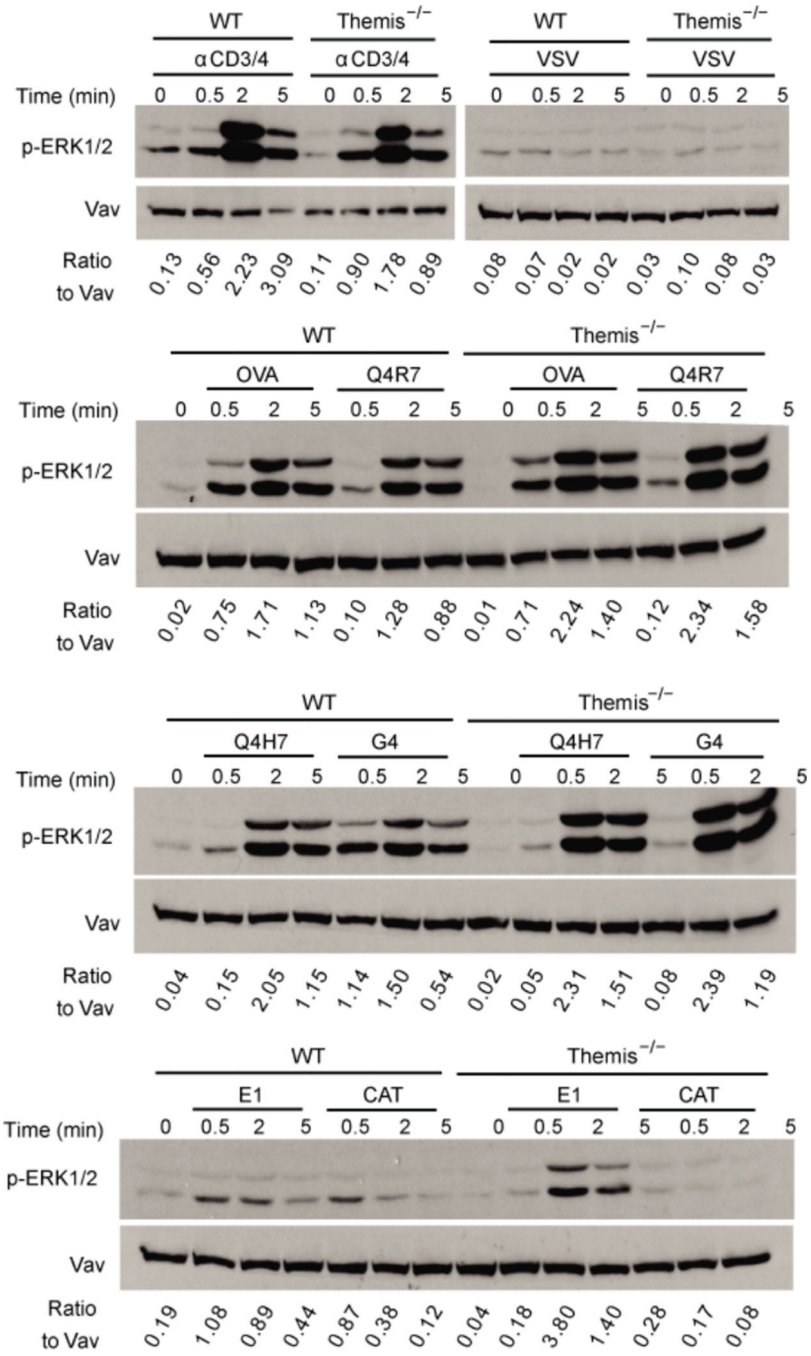
Extended Data Figure 3.

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-peptide tetramers (right). As shown, similar results were obtained by both stimulation methods.



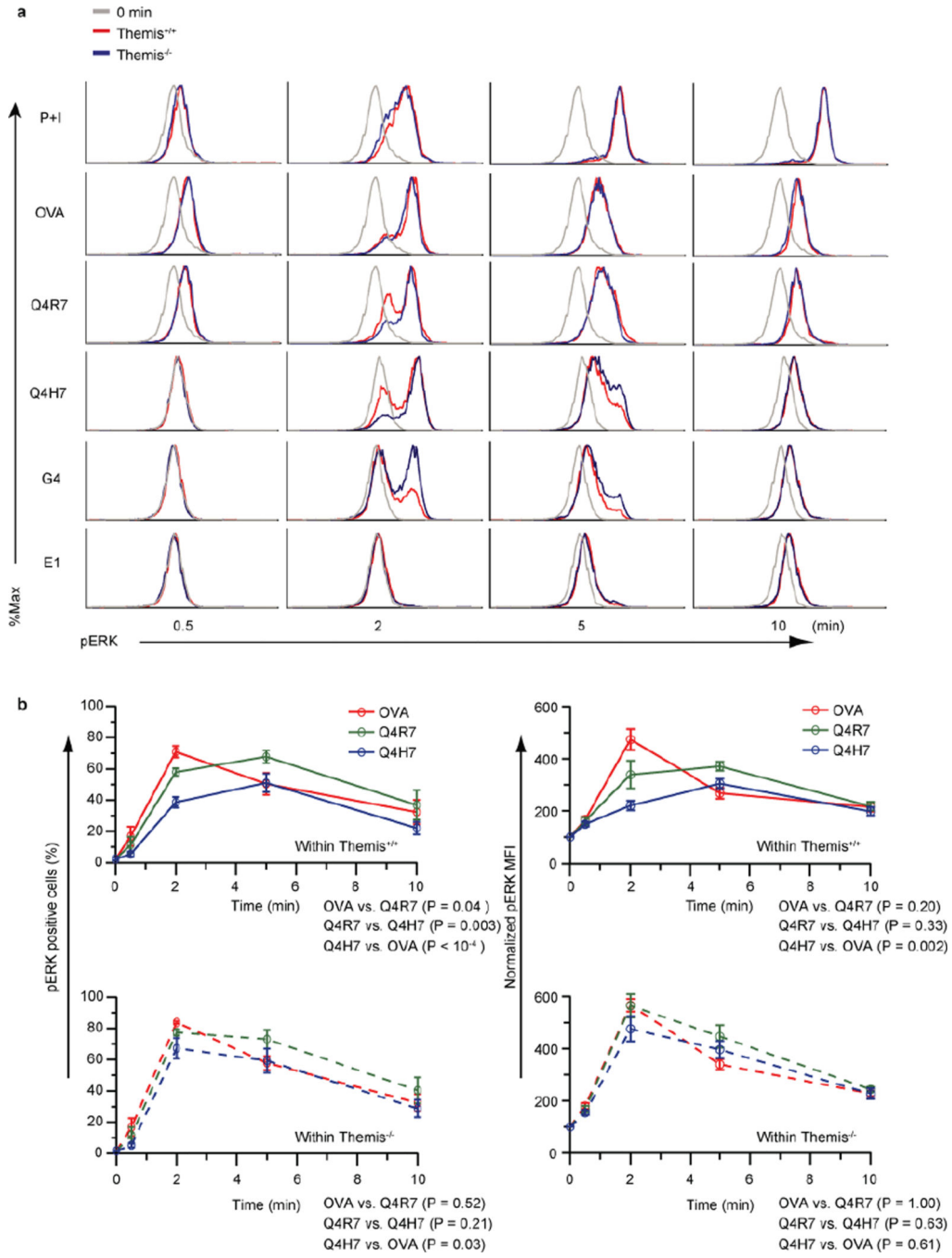
Extended Data Figure 4.

Quantitation of NFAT1 nuclear translocation. Thymocytes were stimulated with Ionomycin/PMA (Iono+PMA) to obtain maximal extent of NFAT1 nuclear translocation as a positive control for image analysis. NFAT1 translocation in non-stimulated cells (CONTROL) is used as negative control for image analysis. DAPI and NFAT staining are color-coded as indicated.



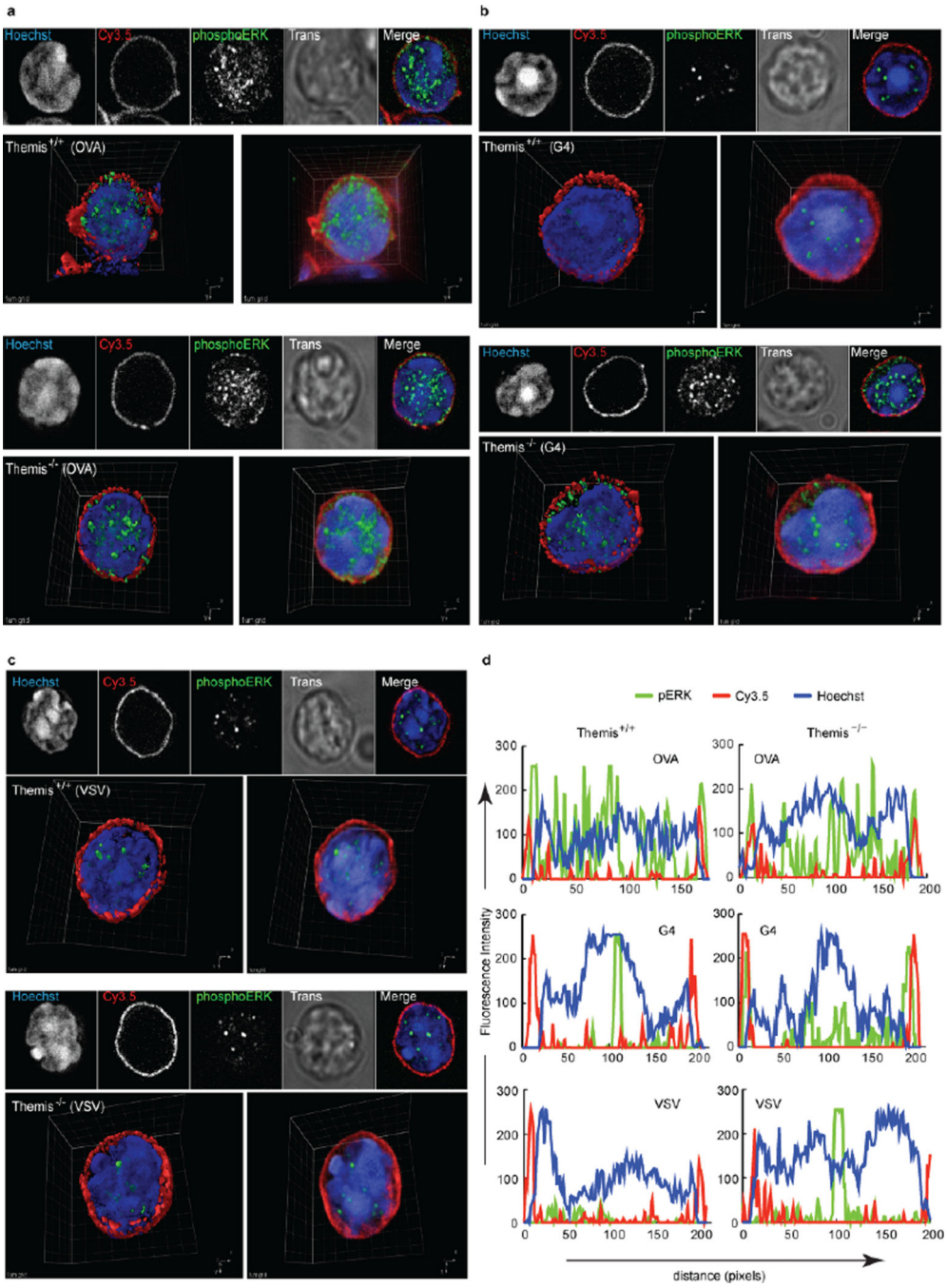
Extended Data Figure 5.

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.

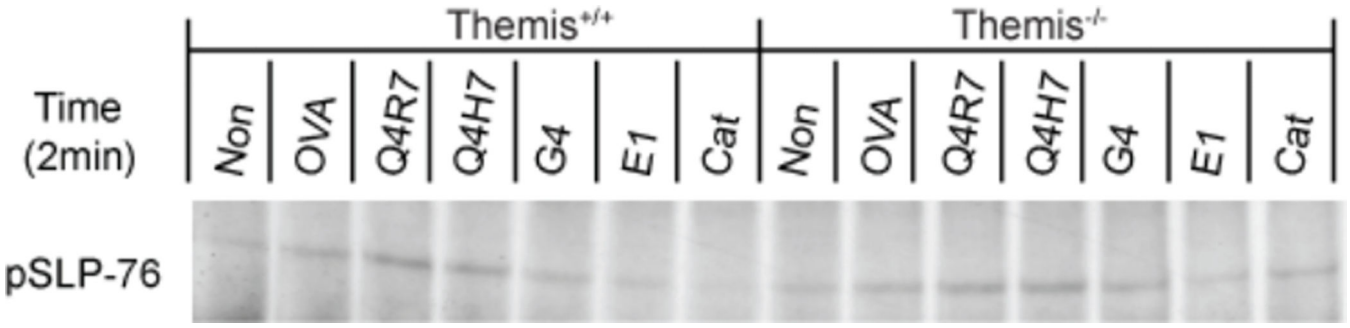
Flow-cytometric analysis of Erk phosphorylation. Thymocytes were prepared and stimulated with K^b-peptide tetramers. Representative FACS plots are shown in (a). Data compiled from several experiments. PMA and ionomycin (P+I) treatment was used as a positive control for stimulation to obtain maximal Erk 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.

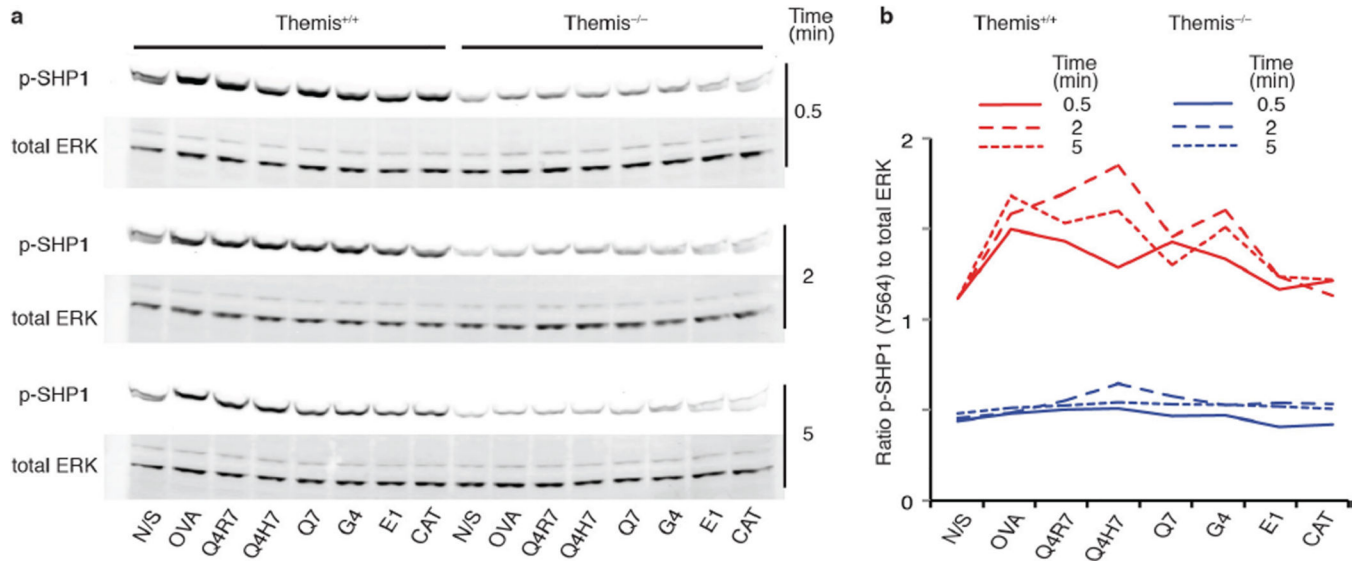
3-dimensional reconstruction images of negative-selection-like Erk signaling in *Themis*$^{-/-}$ thymocytes in response to positive selecting ligands. *Themis*$^{+/+}$ or *Themis*$^{-/-}$ OT-I *Tap*$^{-/-}$ pre-selection thymocytes were stimulated with K^b-OVA (a), K^b-G4 (b) or K^b-VSV (c). Localization of p-Erk was determined by specific staining with anti-p-Erk antibody. Nuclei were counterstained with Hoesch 33342, and plasma membrane was labeled with Cy3.5. Top panels represent each separate channel of a single centered 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 (Hoesch) and red line (Cy3.5).



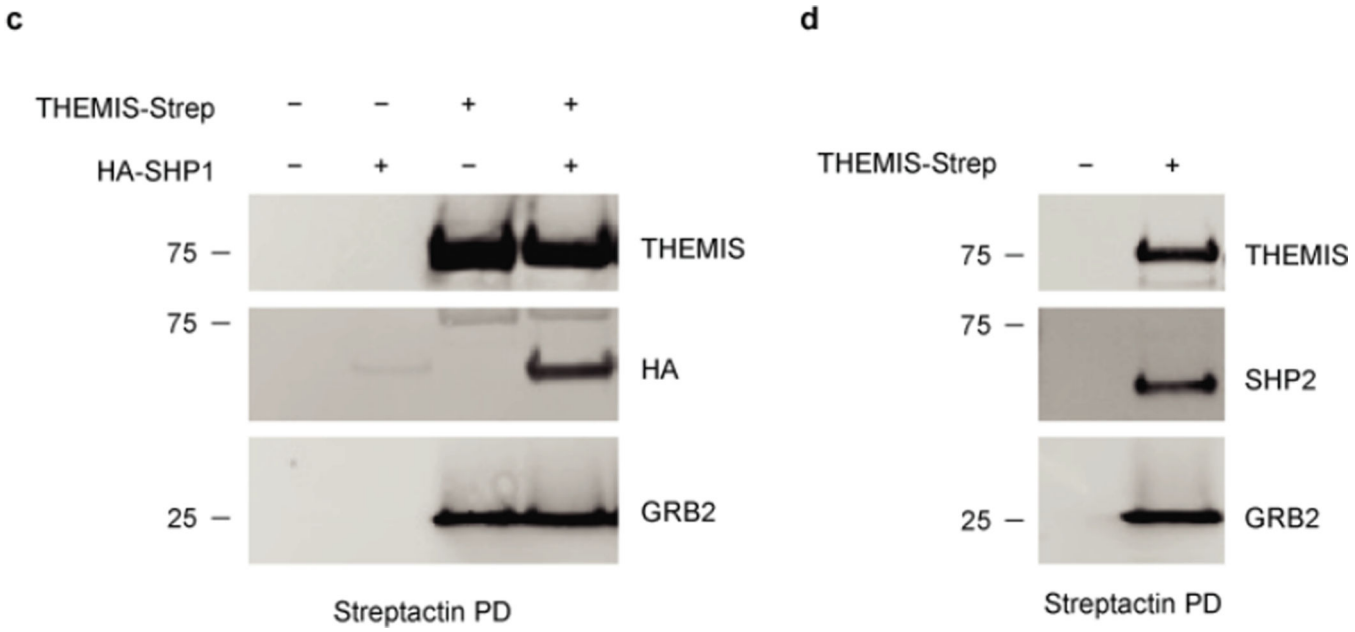
Extended Data Figure 8.

SLP-76 phosphorylation is not affected in Themis-deficient thymocytes. Representative of 2 experiments.



Extended Data Figure 9. Decreased SHP1 phosphorylation in *Themis*$^{-/-}$ DP cells

(a) Phosphorylation of SHP1 was determined in cell lysates. In this separate 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 pSHP1 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. Pull-down assays using Streptactin beads were performed 2 days after transfection and the precipitate subjected to SDS-PAGE and immunoblotting with anti-HA tag (a) and anti-SHP2 (b) antibodies, respectively. Representative of (a) 3 and (b) 2 similar experiments. Note that HEK293 cells express SHP2 but little SHP1. Cells originally from ATCC, tested negative for mycoplasma within previous 3 months, not STR profiled. Constitutive binding of GRB2 to THEMIS has been reported previously.