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## Themis controls thymocyte selection through regulation of T cell receptor-mediated signaling

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### Abstract

Themis (Thymocyte expressed molecule involved in selection), a member of a family of proteins with unknown functions, is highly conserved among vertebrates. Here we found that Themis is expressed in high amounts in thymocytes between the pre-T cell receptor (TCR) and positive selection checkpoints, and in low amounts in mature T cells. Themis-deficient thymocytes exhibit defective positive selection, which results in reduced numbers of mature thymocytes. Negative selection is also impaired in Themis-deficient mice. A higher percentage of Themis-deficient T cells exhibit CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory and CD62L<sup>lo</sup>CD44<sup>hi</sup> memory phenotypes than in wild-type mice. Supporting a role for Themis in TCR signaling, this protein is phosphorylated quickly after TCR stimulation, and is needed for optimal TCR-driven Ca<sup>2+</sup> mobilization and Erk activation.

### INTRODUCTION

T cell development in the thymus follows an ordered progression from CD4<sup>-</sup>8<sup>-</sup> (double negative, DN, subdivided into stages DN1-4) through CD4<sup>+</sup>8<sup>+</sup> (double positive, DP) to single positive (SP, CD4<sup>+</sup>8<sup>-</sup> or CD8<sup>+</sup>4<sup>-</sup>) stages that depends on a complex interplay of signaling pathways<sup>1,2</sup>. In DN3 cells, VDJ rearrangement and expression of the T cell

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receptor (TCR)  $\beta$ -chain and its association with a pre-TCR $\alpha$  chain results in signaling that drives cells through the  $\beta$ -selection checkpoint into the TCR<sup>lo</sup> DP stage. Here, V $\alpha$ -J $\alpha$  rearrangement occurs and a mature  $\alpha\beta$  TCR is expressed on the cell surface. Sequential rearrangements that delete previous rearrangements are frequently required before a TCR is produced that has sufficient ability to interact with self MHC-peptide (pMHC) complexes to induce the positive selection differentiation program that leads to SP thymocyte differentiation<sup>3</sup>. Cells that do not receive this positive selection signal eventually die through lack of stimulation, whereas those cells whose TCR binds too strongly to self pMHC undergo activation induced apoptosis referred to as negative selection<sup>1,2</sup>.

Signaling through the TCR is tightly regulated during positive selection. Pre-selection DP cells express about 10% of the amount of cell surface TCR on post-selection TCR<sup>hi</sup> DP and SP cells, yet the pre-selection DP cells are more sensitive to TCR stimulation by weak ligands than are the TCR<sup>hi</sup> cells<sup>4,5</sup>. The low TCR expression on pre-selection DP cells is actively maintained by Src-family protein tyrosine kinase (PTK) signaling and ubiquitination-mediated degradation<sup>6,7</sup>, and these controls enforce TCR  $\alpha$ -chain allelic exclusion<sup>8,9</sup>. Development through the positive selection checkpoint can be disrupted by mutation of elements of the TCR signaling cascade, such as the ZAP-70, Itk, Lck or Fyn kinases or the Vav guanine nucleotide exchange factor<sup>10,11</sup>. The regulation of signaling through the MAP kinase Erk is extremely important in distinguishing between positive and negative selection<sup>12–15</sup>. Similarly, mobilization of Ca<sup>2+</sup> in response to TCR stimulation is both regulated and related to the discrimination between positive or negative selection<sup>4,16,17</sup>.

Here we identified a novel gene and protein involved in thymocyte positive and negative selection. Two other groups have also identified this gene and protein, and in consensus we refer to it as thymocyte expressed molecule involved in selection (Themis)<sup>18,19</sup>. It is expressed in a tightly regulated manner during T cell development. It is predominantly expressed in late DN and DP thymocytes, and is down-regulated after positive selection. Themis is tyrosine phosphorylated within seconds after TCR stimulation. It is a member of a small gene family of unknown function, and is highly conserved amongst vertebrates. It has no known conserved domains other than a polyproline region. We showed that Themis plays an important role in regulating thymocyte development through TCR signaling and in particular through regulation of Ca<sup>2+</sup> influx and phosphorylation of Erk.

## RESULTS

### ***Themis* encodes a novel protein**

A cDNA subtraction library was constructed using cDNA from *Tcra*<sup>-/-</sup> thymus subtracted with cDNA from *Rag1*<sup>-/-</sup> thymus<sup>20</sup>. A clone which exhibited differential expression between *Tcra*<sup>-/-</sup> and *Rag1*<sup>-/-</sup> thymocyte mRNAs was analyzed further (Fig.1). Full-length cDNA clones were isolated from a mouse thymocyte library and from a human Jurkat cell library. The main open reading frame of the mouse *Themis* gene encodes a protein of molecular weight 72.8 kDa. The human THEMIS protein is 81% identical (86.4% similarity) to the mouse protein (Fig.1a). The gene is present in public databases (Mouse gene #NM\_178666, RIKEN cDNA E430004N04Rik; human NM\_001010923), and

orthologs are found across the mammals, and in birds (*Gallus*) and bony fish (*Danio*) (Supplementary Fig.1).

Although Southern blot experiments showed only a single *Themis*-hybridizing band in mouse genomic DNA, suggesting a single copy gene with no close relatives (data not shown), BLAST analysis of the mouse genome revealed two paralogs: the “basement-membrane-induced” gene *Icb-121*, and the hypothetical protein 9130404H23Rik. *Themis* is more closely related to *Icb-1* (32% identity) than to 9130404H23Rik (25% identity) (Supplementary Fig.2). The mouse *Themis* gene is on chromosome 10, location A4, and human *THEMIS* is at Chr 6q22.33 (C6orf190). The sequences of *Themis*, *Icb-1* and 9130404H23Rik revealed no identifiable conserved domains by PFAM or SMART searches, but there is a highly conserved proline rich sequence (PRS) forming three overlapping PXXP putative SH3 domain-recognition motifs near the C-terminus (residues 555–563) (Fig.1a; all numbering refers to mouse protein). The PRS is conserved in *Icb-1* and 9130404H23Rik (Supplementary Fig.2). A putative bipartite nuclear localization signal lies between residues 330–346.

### ***Themis* gene expression in T cell development**

*Themis* gene expression was analyzed by Northern blot of different tissues (Fig.1b). *Themis* is expressed in the thymus and to a lesser extent in the spleen, but was not detectable in non-lymphoid tissues. Of the two major transcripts, the 5.7 kb transcript was the most abundant. It was readily detectable in a number of transformed cell lines of thymic origin as well, but was undetectable in transformed B cell lines (Supplementary Table 1). Reference to a public gene expression atlas database (biogps.gnf.org)<sup>22</sup> confirmed its very restricted expression pattern, in that it was detected in high amounts in thymus, lower amounts in mature T cells, and very low amounts elsewhere. Remarkably, *Icb-1* expression is restricted to B cells, macrophages and dendritic cells, and 9130404H23Rik is specifically expressed in the intestine (Supplementary Fig.3).

Real-time RT-PCR analysis of thymocyte subsets showed that *Themis* expression was low in DN1 and DN2 cells and was upregulated in DN3 cells; expression remaining high in TCR<sup>low</sup> immature pre-selection DP thymocytes, and was downregulated in post-positive selection TCR<sup>int</sup> and TCR<sup>hi</sup> cells (Fig.1c). *Themis* expression remained low in mature SP subsets. Positive selection is also marked by the migration of immature thymocytes from the thymus cortex towards the medulla, where only mature SP thymocytes that have survived the selection process are found. *In situ* hybridization showed that *Themis* expression was detectable mainly in cortical thymocytes with little expression in medullary thymocytes (Fig.1d). Thus, *Themis* expression is high in immature cortical thymocytes but low in mature medullary thymocytes.

These data suggested that *Themis* expression may be downregulated by stimulation through the  $\alpha\beta$  TCR. To explore this possibility we used the OT-I TCR transgenic (tg) mouse, which expresses a TCR recognizing a peptide derived from ovalbumin (OVA). A set of peptides is available with different affinities for the OT-I TCR, and positive and negative selection of OT-I TCR tg thymocytes is blocked in mice lacking MHC class I expression (e.g. *Tap1*<sup>-/-</sup>)<sup>15,23–25</sup>. We therefore injected OT-I *Tap1*<sup>-/-</sup> mice (whose thymocytes are blocked

at the pre-selection DP stage<sup>25</sup>) with peptides to stimulate the TCR *in vivo*<sup>26</sup>. Injection of antigenic OVA peptide, but not a non-stimulatory VSV control peptide, resulted in downregulation of *Themis* expression (Fig.1e). Basal *Themis* expression in Tap1-expressing thymocytes was lower than in Tap1-deficient cells, presumably due to the *Themis* downregulation induced by positive selection. These data indicate that stimulation through the TCR by pMHC induces downregulation of *Themis*.

### Defective positive selection of *Themis*<sup>-/-</sup> thymocytes

To determine the function of *Themis* in T cell development, we generated *Themis*-deficient mice. A construct targeting the first exon of *Themis*, replacing it with a neomycin cassette, was prepared and transfected into 129Sv ES cells (Supplementary Fig.4). These ES cells were screened, selected and injected into blastocysts by standard methods. One progeny was >90% chimeric and was bred. *Themis*<sup>+/-</sup> offspring mice were backcrossed to B6 for 8 generations and were then intercrossed to generate homozygous mutants (*Themis*<sup>-/-</sup>). *Themis* gene disruption resulted in intermediate or complete loss of expression of *Themis* protein in *Themis*<sup>+/-</sup> or *Themis*<sup>-/-</sup> mice, respectively (Supplementary Fig. 4c).

*Themis*-deficient mice were viable, born at Mendelian frequency, and presented no gross abnormalities. Total thymocyte number was similar to wild-type mice (Supplementary Fig. 5). The proportion and number of DP thymocytes was slightly but significantly increased ( $P < 0.05$ , Student's t-test), while the proportion and number of mature SP cells—particularly CD4 SP cells—was decreased ( $P < 0.01$ ) (Fig. 2a and Supplementary Fig. 5). To identify the developmental block in the *Themis*<sup>-/-</sup> mice, we subdivided thymocytes into five populations on the basis of differential CD3 and CD69 expression and analyzed CD4 and CD8 expression (Fig.2b). Population 1, the most immature TCR<sup>lo</sup>CD69<sup>lo</sup> cells, is composed mostly of DN and DP cells. This population was present in similar proportions in the *Themis*<sup>-/-</sup> and *Themis*<sup>+/+</sup> mice. Population 2, which expresses a TCR<sup>int</sup>CD69<sup>lo</sup> phenotype and corresponds to pre-selection DP cells, was present in slightly higher proportions in *Themis*<sup>-/-</sup> mice, as expected from the larger percentage of DP cells in these mice (Fig.2a). Population 3 partially upregulated CD69 and increased TCR expression relative to population 2, and was present in slightly lower proportions in *Themis*<sup>-/-</sup> mice. Population 4 (TCR<sup>hi</sup>CD69<sup>hi</sup>; post-positive selection thymocytes) was markedly depleted in the *Themis*-deficient thymus. In the wild-type thymus, most of this population was CD4 or CD8 SP, whereas in *Themis*-deficient mice the cells were mostly CD4<sup>+</sup>CD8<sup>int</sup> or CD8 SP. Lastly, *Themis*<sup>-/-</sup> thymi contained very few TCR<sup>hi</sup>CD69<sup>lo</sup> population 5 cells, which correspond to mature SP cells ready for export to the periphery. From these data it is clear that the *Themis*-deficient cells are mostly blocked at the earliest stage of positive selection during which CD8 downregulation is initiated, and that very few *Themis*-deficient thymocytes become fully mature in phenotype.

To establish if *Themis* acts in a T cell-intrinsic manner, we performed a bone marrow (BM) reconstitution experiment (Fig.2c). BM cells from *Themis*<sup>+/+</sup> Thy1.1 Ly5<sup>b</sup> mice and from *Themis*<sup>-/-</sup> Thy1.2 Ly5<sup>b</sup> mice were injected into irradiated Thy1.2 Ly5<sup>a</sup> recipient mice and allowed to reconstitute the thymus for 6 weeks. Ly5<sup>b</sup>-expressing donor cells were gated and then divided into Thy1.1<sup>+</sup> (*Themis*<sup>+/+</sup>) and Thy1.2<sup>+</sup> (*Themis*<sup>-/-</sup>) populations. This flow

cytometric analysis demonstrated that the defect in development from DP to SP cells is intrinsic to the *Themis*<sup>-/-</sup> thymocytes as *Themis*<sup>-/-</sup> thymocytes showed reduced proportions of CD4 SP cells and increased proportions of DP cells relative to the *Themis*<sup>+/+</sup> cells developing in the same environment.

We next analyzed the effect of *Themis*-deficiency on thymocyte development in mice expressing the MHC class II restricted TCR tg AND27, and the class I-restricted OT-I TCR tg24 (Fig.2d,e). The defect in thymocyte development in *Themis*-deficient TCR tg mice was more pronounced than in non-tg polyclonal *Themis*-deficient mice. In AND TCR tg thymi, development was blocked at the DP stage, with very few cells proceeding to CD4 SP (Fig. 2d). The maturation arrest in the *Themis*<sup>-/-</sup> AND thymus was first manifest at the start of the transition from CD4<sup>highhi</sup> to CD4<sup>highint</sup>, with little or no progression to mature SP (Supplementary Fig.6a). In OT-I thymi, the developing class I-restricted cells pass through the CD4<sup>highint</sup> stage prior to upregulating CD8 and down-regulating CD4<sup>28</sup>. Strong reduction of this stage in OT-I *Themis*<sup>-/-</sup> thymi (Fig.2e and Supplementary Fig.6b) suggests that, as in AND TCR tg and non-tg mice, *Themis*<sup>-/-</sup> OT-I mice show a strong defect at the earliest stage of positive selection. The architecture of the thymus was altered in *Themis*<sup>-/-</sup> mice, the medulla being smaller and fragmented compared to wild-type thymi (Fig.2f); this defect is indicative of impaired T cell maturation<sup>29</sup>.

### Themis involvement in negative selection

TCR stimulation of DP and SP cells also induces negative selection. Thus, we investigated the role of *Themis* in negative selection in a superantigen-mediated deletion model. The C57BL-derived mouse strains express superantigens Mtv-8 and 9, which cause deletion of V $\beta$ 5<sup>+</sup>, V $\beta$ 11<sup>+</sup> and V $\beta$ 12<sup>+</sup> TCR clonotypic thymocytes when the MHC molecule I-E is also present<sup>30,31</sup>. To introduce I-E, which is absent on the B6-backcrossed *Themis*<sup>-/-</sup> mice, we bred them to B10.D2 mice which express I-E. Comparison of T cell populations in H-2<sup>b/b</sup> (lacking I-E) and H-2<sup>b/d</sup> (expressing I-E) *Themis*<sup>+/-</sup> thymi showed superantigen-mediated deletion of T cells expressing V $\beta$ 5, V $\beta$ 11, and V $\beta$ 12 (Fig.3). V $\beta$ 6<sup>+</sup> (and V $\beta$ 7<sup>+</sup>, not shown) thymocytes do not recognize Mtv-8 and 9, and were not deleted. In fact, their proportion rose slightly in mice where deletion of the other clonotypes had occurred. In *Themis*<sup>-/-</sup> mice, superantigen-mediated deletion was significantly less efficient than in *Themis*<sup>+/-</sup> H-2<sup>b/d</sup> mice, although it was clearly partially active.

We also analyzed thymocyte development in mice expressing the MHC class I-restricted HY TCR tg. Here, positive selection in females leads to a large CD8 SP population, but negative selection predominates in males, resulting in few DP or SP cells<sup>32,33</sup>. HY TCR tg females lacking *Themis* resembled AND TCR tg and OT-I TCR tg *Themis*<sup>-/-</sup> mice in that few thymocytes progressed to become mature CD8 SP (Supplementary Fig.7a). HY TCR tg *Themis*<sup>+/-</sup> males showed a much reduced number of thymocytes compared to HY TCR tg females, and a large proportion of these cells were HY-TCR<sup>+</sup> clonotypic cells expressing low amounts of CD4 and CD8<sup>34,34</sup>. These cells are believed to escape negative selection by loss of the coreceptor. In male HY TCR tg *Themis*<sup>-/-</sup> thymi, negative selection was apparently incomplete in that CD4 and CD8 expression was much lower than normal, but not as low as in the *Themis*<sup>+/-</sup> males, and the clonotype-negative cells found in the

*Themis*<sup>+/-</sup> males were not present (Supplementary Fig.7b). Thus both negative and positive selection were reduced in the absence of Themis, suggesting that absence of Themis leads to a general defect in TCR-mediated signaling.

### Peripheral T cells in *Themis*<sup>-/-</sup> mice

The deficiency in SP thymocytes led to a reduction in peripheral T cell numbers in *Themis*<sup>-/-</sup> mice (Fig.4a,b). The percentage of B cells increased due to the loss of T cells (data not shown). The deficiency in peripheral T cells was most evident in the CD4 population, but both CD4 and CD8 cells were significantly reduced in number. Analysis of TCR tg mice confirmed these data, with very few mature peripheral CD4 cells in AND *Themis*<sup>-/-</sup> (Fig.4c), and few CD8 cells in OT-I *Themis*<sup>-/-</sup> (Fig.4d) TCR tg mice. Within the CD4<sup>+</sup> population, cells with a regulatory T (T<sub>reg</sub>) cell phenotype (CD25<sup>+</sup>Foxp3<sup>+</sup>) were increased in frequency from 10% in *Themis*<sup>+/+</sup> to 21% in *Themis*<sup>-/-</sup> (Fig.4e), though the absolute number of *Themis*<sup>-/-</sup> T<sub>reg</sub> cells was ~50% of the number in *Themis*<sup>+/+</sup> mice (data not shown).

The expression of CD62L and CD44 in peripheral T cells is used to differentiate naïve from memory T cells. There was a lower percent of CD62L<sup>hi</sup>CD44<sup>lo</sup> naïve phenotype cells, and a higher proportion of CD62L<sup>lo</sup>CD44<sup>hi</sup> memory phenotype cells in *Themis*<sup>-/-</sup> mice (Fig.4f). Expression of CD122<sup>+</sup> (interleukin 2Rβ) was also higher in *Themis*<sup>-/-</sup> mice (data not shown). Thus the few peripheral cells in Themis-deficient mice have a phenotype redolent of memory cells, a frequent observation in partially lymphopenic mice that probably reflects homeostatic T cell population expansion in the periphery<sup>35</sup>.

We then tested the ability of *Themis*<sup>-/-</sup> spleen T cells to respond to anti-CD3 stimulation (Fig.5). CD69 upregulation was strongly reduced in Themis-deficient CD4 and CD8 T cells compared to wild-type T cells. Proliferation of both CD4 and CD8 *Themis*<sup>-/-</sup> T cells in response to anti-CD3 was very poor compared to *Themis*<sup>+/+</sup> T cells. *Themis*<sup>+/+</sup> and *Themis*<sup>-/-</sup> CD8 T cells expressed similar amounts of surface TCR, but TCR expression was slightly reduced on the surface of *Themis*<sup>-/-</sup> CD4 cells (Fig.5a).

### Themis in early TCR-signaling

The role of Themis in the TCR signaling cascade was investigated by immunoprecipitation and immunoblotting (Fig.6). Initially, a proteomic study of global dynamics of TCR-directed tyrosine phosphorylation using stable isotopic labeling of amino acids in cell culture (SILAC), identified a number of novel potential signaling components in Jurkat cells, including C6orf190 (C.B., O.A., M.S., to be published elsewhere), which was named SPoT (for Signaling Phosphoprotein specific of T cell, which we refer to here as THEMIS). Reduction of THEMIS expression by siRNA in Jurkat cells resulted in substantial decrease of interleukin 2 (IL-2) production induced by anti-TCR and anti-CD28 stimulation (C.B., O.A., M.S., unpublished data). SILAC data suggested that THEMIS was phosphorylated on tyrosine as early as 30 seconds after TCR stimulation and that this phosphorylation was reduced at 5 minutes after TCR stimulation; these phosphorylation kinetics are similar to those of other known TCR-proximal signaling proteins (e.g., LAT, SLP-76) (data not shown).



To corroborate these data, Jurkat cells were stimulated with anti-CD3, lysates were immunoprecipitated with anti-phosphotyrosine (pTyr), and immunoprecipitates were incubated with an antiserum against the C-terminal sequence of THEMIS. This treatment revealed a phosphoprotein of the expected size for THEMIS (~72 kD, Fig.6a) that increased in abundance after 30 seconds of stimulation then declined. Immunoprecipitation with anti-THEMIS and immunoblotting with anti-pTyr provided direct evidence that THEMIS was itself phosphorylated on a tyrosine residue, indicating that it is a TCR-dependent PTK substrate. Similar data were obtained with human CD4<sup>+</sup> PBL T cells (Fig.6b). In mouse thymocytes, Themis was tyrosine phosphorylated within 30 seconds after TCR crosslinking, and phosphorylation was undetectable after 3 minutes (Fig.6c). Together these data show that in mouse and human cells Themis is an early target of TCR-controlled PTK(s) and suggest that it is a component of the TCR signaling pathway whose function and-or recruitment may depend on tyrosine phosphorylation.

### Defective TCR signaling in *Themis*<sup>-/-</sup> thymocytes

Because the proline-rich sequence in Themis and related proteins is predicted to be a binding site for SH3 domains, we tested an SH3 domain array to screen for binding to the N-terminal and C-terminal (including the PRS) halves of Themis. The PRS-containing region bound significantly to the phospholipase C- $\gamma$ 1 (PLC- $\gamma$ 1) SH3 domain (data not shown). To confirm a Themis-PLC $\gamma$ 1 interaction in thymocytes, we stimulated B6 thymocytes with anti-CD3, then immunoprecipitated cell lysates with Themis-specific antiserum. Although there was some interaction between Themis and PLC- $\gamma$ 1 prior to stimulation, the amount of PLC $\gamma$ 1 coprecipitated with Themis increased at 2 minutes post-stimulation, but ultimately returned to a weaker interaction (Fig.6d). As Itk is a Tek-family PTK that is required for activation of PLC- $\gamma$ 1 and for TCR-signaling<sup>36</sup>, we tested the possibility of an interaction between Itk and Themis. Anti-Itk coprecipitated both Themis and PLC- $\gamma$ 1 from freshly isolated, anti-CD3-stimulated thymocytes (Fig.6e). It appeared that that Itk-Themis and Itk-PLC- $\gamma$ 1 interactions increased after CD3 crosslinking. To test this, we rested thymocytes in culture for 2h before activation with anti-CD3 plus anti-CD4. As previously reported<sup>37</sup>, PLC- $\gamma$ 1 interacted constitutively with Itk in thymocytes; after stimulation the interaction increased slightly before declining after 30 seconds (Fig.6f). Interaction of Themis with Itk was not constitutive, but was induced by 30 seconds after TCR stimulation.

To determine which signaling pathways are defective in *Themis*<sup>-/-</sup> thymocytes, we analyzed immunoblots of anti-CD3 plus anti-CD4 activated thymocytes from *Themis*<sup>+/+</sup> and *Themis*<sup>-/-</sup> thymocytes (Fig.6g). We found no difference in ZAP70, PLC- $\gamma$ 1 or Itk (or LAT, data not shown) phosphorylation in response to TCR stimulation. However, Erk showed reduced phosphorylation in *Themis*<sup>-/-</sup> cells. Erk1 phosphorylation (p-p44) was delayed in *Themis*<sup>-/-</sup> compared to *Themis*<sup>+/+</sup> cells, and Erk2 phosphorylation (p-p42) was reduced in *Themis*<sup>-/-</sup> cells at 30 and 90 seconds after stimulation (Fig.6g, Supplementary Fig.8a), and was not always detectable at 3 minutes. This experiment was performed three times with similar results (Supplementary Fig.8b). Phosphorylation of Raf, Mek and Jnk was not affected by Themis deficiency (data not shown).

To look at other aspects of TCR signaling, we stimulated pre-selection DP thymocytes from OT-I TCR tg *Tap1*<sup>-/-</sup> mice. Stimulation by anti-CD3 plus anti-CD4 or OVA-K<sup>b</sup> tetramers (Fig.7a) induced a slightly slower and reduced initial peak of store-operated Ca<sup>2+</sup> influx in *Themis*<sup>-/-</sup> cells relative to *Themis*<sup>+/+</sup> cells. This difference in Ca<sup>2+</sup> influx, while relatively subtle, was very reproducible (a total of 9 *Themis*<sup>-/-</sup> versus 5 *Themis*<sup>+/+</sup> in 3 separate experiments). The release of endoplasmic reticulum Ca<sup>2+</sup> stores was very low in the thymocytes, and we found no difference between *Themis*<sup>-/-</sup> cells relative to *Themis*<sup>+/+</sup> cells.

Organization of the cytoskeleton at the immunological synapse is regulated by TCR signaling<sup>38</sup>. We therefore imaged actin polymerization in OT-I *Tap1*<sup>-/-</sup> thymocytes expressing or lacking Themis as they interacted with OVA peptide presented by EL4 tumor cells (Fig.7b). The actin polymerization pattern in Themis-sufficient thymocytes was similar to that in OT-I lymph node T cells, but Themis-deficient thymocytes showed a significant defect in actin polymerization at the immunological synapse. A useful correlate of stimulation of thymocytes through the TCR by positive or negative selecting ligands is upregulation of CD69<sup>15,25,39,40</sup>. We found that the *Themis*<sup>-/-</sup> OT-I *Tap1*<sup>-/-</sup> pre-selection DP thymocytes were less able to upregulate CD69 in response to OVA peptide than their *Themis*<sup>+/+</sup> OT-I *Tap1*<sup>-/-</sup> counterparts (Fig.7c). These data suggest that Themis is involved in regulating the TCR-signaling cascade, albeit with relatively small effects on early events in the cascade.

## DISCUSSION

Development of αβ T cells is critically dependent on signaling through the TCR, and can be disrupted by deficiencies in the receptor itself and many signaling molecules and mediators. In this work, we identified a novel protein, Themis, that is required for passage through the positive selection checkpoint.

Themis deficiency results in a defect in development that is stronger in CD4 cells than in CD8 cells. However, this defect was evident in both MHC class I and class II-restricted TCR transgenic lines, wherein thymocyte development was severely blocked at the earliest stages of positive selection. A stronger phenotype in TCR tg thymocytes is a common finding in mice deficient in signaling molecules, as the normal TCR repertoire is plastic enough to select for TCRs with higher or lower affinity, thereby allowing the signal strength to be modulated to permit selection<sup>10</sup>. The strong developmental block in the TCR tg *Themis*<sup>-/-</sup> mice, together with the evidence indicating that negative selection signaling is also deficient in *Themis*<sup>-/-</sup> mice, suggested a role for Themis in the TCR signaling cascade rather than in a developmental program *per se*. Because Themis is expressed in immature thymocytes, and is downregulated by stimulation through the TCR as occurs during positive selection, the signaling role of Themis is likely to be most important in DP thymocytes before or during positive selection. In agreement with this notion, *Themis*<sup>-/-</sup> mice pre-selection thymocytes responded poorly compared to *Themis*<sup>+/+</sup> thymocytes to TCR stimulation, as measured by CD69 upregulation. *Themis*<sup>-/-</sup> thymocytes also showed reduced actin polymerization at the immunological synapse, as in *Itk*-deficient and *Vav*-deficient cells. These results are consistent with a defect in TCR signal transduction<sup>39–42</sup>.



Themis was tyrosine phosphorylated within 30 seconds of TCR stimulation, suggesting that it may play a role early in signal transduction through the TCR. Moreover, it interacted inducibly with Itk and PLC- $\gamma$ 1, although phosphorylation of these proteins was not altered in *Themis*<sup>-/-</sup> cells. Deletion of Itk has a profound effect on Ca<sup>2+</sup> signaling, but a relatively minor effect on thymocyte development<sup>36,41</sup>. In contrast, Themis-deficiency had a strong effect on development but a small effect on Ca<sup>2+</sup> signals (e.g. slightly slower and weaker Ca<sup>2+</sup> flux). The effect of loss of Themis on Erk signaling was also quite subtle, and manifested as a reduction in the strength and duration of phosphorylation. These findings suggest that Themis might negatively regulate phosphatases that turn off Erk signaling. Notably, similarly small differences in p-Erk induction distinguished signals that induce positive and negative selection of thymocytes<sup>14,15</sup>, and sustained Erk signals are required for positive selection<sup>2,12,14</sup>. Additionally, there is evidence that slight changes in Ca<sup>2+</sup> signaling lead to profound differences in MAP kinase activation in non T cells<sup>43</sup>, and in pre-selection thymocytes, and that these differences distinguish the quality of TCR stimulation<sup>17</sup>. The alterations in Ca<sup>2+</sup> and Erk signaling between *Themis*<sup>-/-</sup> and *Themis*<sup>+/+</sup> pre-selection thymocytes suggest that Themis may be involved in setting the threshold for TCR responses to selecting signals. Such a role for Themis can account for its expression primarily in pre-positive selection thymocytes, and would indicate that its role in TCR-mediated signaling may not be so important in peripheral T cell activation. Study of the normal role of Themis in peripheral T cell signaling will have to await generation of a conditional *Themis*<sup>-/-</sup> mouse.

When given the opportunity to produce a full TCR repertoire, some Themis-deficient thymocytes pass through the positive selection checkpoint and generate peripheral T cells. These cells respond poorly to TCR-mediated stimulation. Interestingly, in both CD4 and CD8 subsets, there was an increased prevalence of memory-phenotype (CD44<sup>hi</sup>CD62L<sup>lo</sup>CD122<sup>+</sup>) cells and reduced proportions of naïve-phenotype populations. This may be the result of homeostatic population expansion in a lymphopenic environment<sup>35</sup>, and is similar to the phenotype seen in mice lacking Itk. Memory phenotype T cells in Itk-deficient mice have been referred to as “innate-like” T cells<sup>44</sup>. This phenotypic similarity to Itk-deficient mice supports the notion that Themis is involved in TCR signal transduction via Itk.

We also noted a strongly increased proportion (though reduced absolute number) of T<sub>reg</sub> cells in *Themis*<sup>-/-</sup> mice. Interestingly the *Themis* gene was identified as one of the transcripts most strongly downregulated by Foxp3 in response to TCR stimulation<sup>45</sup>, and its suppression may therefore be part of the T<sub>reg</sub> cell development program.

Themis, Icb-1 and 9130404H23Rik are members of a small and essentially uncharacterized family. Each has a very restricted cell-type-specific expression and is highly conserved in vertebrates. Other than a PRS, they have no predicted conserved domains that are shared with other proteins. These properties point to the family having important and perhaps novel functions and structure. Here we showed that one of these family members, Themis, is a T cell specific protein with a crucial function in positive selection through transducing TCR signals in thymocytes.

## Accession Codes

Mouse *Themis*: MGI:2443552, Human *THEMIS* GeneID: 387357.

## METHODS

### Mice

*Tcra*<sup>-/-</sup> (B6.129S2-*Tcra*<sup>tm1Mom/J</sup>), *Rag1*<sup>-/-</sup> (B6.129S7-*Rag1*<sup>tm1Mom/J</sup>), and Ly5<sup>a</sup> congenic mice (B6.SJL-*Ptprc*<sup>a</sup> *Pep3*<sup>b</sup>/BoyJ) on the B6 background were obtained from Jackson Labs. Other strains were bred at The Scripps Research Institute (TSRI) and maintained in accordance with TSRI Animal Care and Use Committee.

### Antibodies

Antibodies recognizing CD3 (145-2C11), CD4 (RM4.4, RM4.5, GK1.5), CD8 $\alpha$  (53-6.7), CD25 (PC61.5), CD62L (MEL14), CD44 (IM7), Itk (2F12), PLC- $\gamma$ 1 (10/PLCg), Foxp3 (FJK-165), V $\beta$ 5 (MR9-6), V $\beta$ 6 (RR4-7), V $\beta$ 11 (CTVB11), and V $\beta$ 12 (CTVB12b), were obtained from eBiosciences or BDBiosciences. Anti-p-Tyr (4G10) was from Upstate Biotechnology. Anti-p42/44 (3A7) and p-p42/44 (197G2) and antisera to p-ZAP70 and p-PLC- $\gamma$ 1 were from Cell Signaling Technology. Anti-*Themis* was raised in rabbits against the N-terminal 294 amino acids, made as a fusion to glutathione-S-transferase (GST), purified and with the GST removed. The antiserum was affinity purified. Anti-*THEMIS* serum was made by immunizing rabbits with a peptide corresponding to the C-terminal 15 amino acids of the human sequence. Specificity is demonstrated in Supplementary Fig.9.

### Cloning of *Themis*

A subtraction library was constructed from cDNA from *Tcra*<sup>-/-</sup> thymus mRNA subtracted with cDNA from *Rag1*<sup>-/-</sup> thymus mRNA as described<sup>20</sup>. Clones randomly picked from the library were sequenced and used to search Genbank to identify potentially novel clones. A full length clone of human *THEMIS* was isolated by screening a Jurkat cDNA library.

### Real-time RT-PCR Analysis

Real-time PCR was performed using SYBERgreen PCR Master Mix and the Prism 7900 instrument and analyzed with SDSv2.1 software (Applied Biosystems). Results were normalized to  $\beta$ -actin expression. Primer sequences are shown in Supplementary Table 2).

### Generation of *Themis*-deficient mice

Exon 1 of *Themis* was replaced by the 1.6 kb PGK neomycin cassette. The target vector (pKO Scrambler) contained the flanking sequence of exon 1: a 5' 2.8 kb short arm and a 3' 3.9 kb long arm sequence flanking the neomycin phosphotransferase gene. Embryonic stem cells (129SvEv strain) were electroporated with 25 $\mu$ g linearized DNA/10<sup>7</sup> cells, and cultured for 7d in medium containing 400  $\mu$ g/ml G418. Drug resistant clones were expanded and homologous recombination confirmed by PCR and Southern blot. Positive clones were injected into blastocysts derived from B6 embryo donor mice mated with C6D2F1 stud males (B6  $\times$  DBA2/J hybrids) and transplanted into pseudopregnant CD-1 recipients.

Chimeras were bred to C57BL/6J females in order to reveal germ-line transmission followed by backcrossing to B6 (10 generations).

### Calcium flux

Thymocyte suspensions were prepared and split into separate tubes. Part was loaded with CFSE (20 nM, for 10 min at 37°C) or Cy5 (1µg/ml, 5min RT), or mock treated, followed by washing. CFSE-labeled and/or Cy5-labeled populations were mixed equally with a mock-loaded sample, for example; OT-I *Tap1*<sup>-/-</sup>*Themis*<sup>+/+</sup> (mock) plus OT-I *Tap1*<sup>-/-</sup>*Themis*<sup>-/-</sup> (CFSE or Cy5). In some experiments, the CFSE- and Cy5-treated and untreated pairs were reversed to ensure that the CFSE or Cy5 did not alter the results. Cells were suspended at 10<sup>7</sup>/ml in cRPMI (RPMI supplemented with 10% FCS, 100U/ml Penicillin, 10µg/ml Streptomycin, 292µg/ml Glutamine, 50µM 2-ME, 25mM HEPES) and were incubated with 2µM Indo-1 AM (Molecular Probes) for 30 min at 37°C, 5% CO<sub>2</sub>. Cells were washed twice with cRPMI. Cells were treated with biotin-anti-CD3 and anti-CD4 as well as PerCPCy5.5-anti-CD8 and PECy7-anti-CD4 on ice for 20min. Cells were washed once with cRPMI and once with Ca<sup>2+</sup> free medium (cHBSS: Ca<sup>2+</sup> and Mg<sup>2+</sup> free HBSS supplemented with 1% FCS, 1mM MgCl<sub>2</sub>, 1mM EGTA, and 10mM HEPES), and resuspended in cHBSS. Cells were pre-warmed to 37°C before analysis and were kept at 37°C during event collection on a Becton-Dickinson LSRII. To stimulate cells, 10µg/ml Streptavidin (Jackson) was added to cross-link biotinylated antibodies; alternatively, cells were stimulated with tetramers made as described from refolded K<sup>b</sup> and β2m15,23, or purchased from Beckman-Coulter (iTag MHC tetramer). 5mM CaCl<sub>2</sub> was added during analysis, and maximal Ca<sup>2+</sup> flux obtained by adding 500ng/ml of ionomycin (Calbiochem). Mean fluorescence ratio was calculated using FlowJo (TreeStar).

### T cell activation and proliferation assay

Lymphocytes from *Themis*<sup>+/+</sup> (Thy1.1, B6.PL) or *Themis*<sup>-/-</sup> (Thy1.2, B6) mice were mixed 1:1. Cells were cultured in the presence of soluble anti-CD3 for 5h, stained with anti-CD69 and anti-Thy1 allotypes and analyzed by FACS. For proliferation assays, cells were stained with 0.2µM CFSE at 37°C for 10 min. Staining was stopped by adding an equal volume of FCS. After washing, cells were cultured with or without stimulants (e.g. anti-CD3) in cRPMI for 48h. T cell proliferation was measured by CFSE dilution analyzed by FACS.

### Thymocyte CD69 upregulation assay

Thymocytes (2–3×10<sup>5</sup>) from OT-I tg *Tap1*<sup>-/-</sup> mice (*Themis*<sup>+/-</sup> or <sup>-/-</sup>) were incubated with 2×10<sup>5</sup> peptide-pulsed EL4 cells in round-bottom 96-well plates at 37° for 5h. Cells were stained and analyzed by FACS as described<sup>39,40</sup>. Gates defining CD69<sup>-</sup> versus CD69<sup>+</sup> were determined from thymocytes incubated with non-peptide pulsed EL4 cells.

### Immunoprecipitation and immunoblotting

Cells were lysed in buffer containing 20mM Tris pH7.5, 150mM NaCl, 0.5% n-Dodecyl-β-D-maltoside or NP40, 20mM NaF, 1mM Na<sub>3</sub>VO<sub>4</sub>, and protease inhibitor cocktail (Roche). Immunoprecipitation was performed on cell lysates (0.5–1 mg of protein) overnight at 4°C, then protein-A sepharose beads (Amersham) were added for 1h. After washing, samples

were resolved on 4–12% SDS-PAGE and blotted. The membrane was blocked 1h with PBS, 1% TWEEN, 5% dry milk, incubated with primary Ab for 3h at room temperature or overnight at 4°C, washed and incubated with the secondary Horseradish peroxidase-antibody for 1.5h followed by enhanced chemiluminescence detection of signal (Pierce).

### In situ hybridization

Thymi from 3–4 week-old mice were fixed, embedded in paraffin and 5µm sections probed with antisense <sup>35</sup>S-UTP-labeled *Themis* RNA. Sections were RNAase A-treated, washed (0.2X SSC, 42°C), dried, coated with Kodak NTB2 emulsion, exposed for 1–2 weeks, developed, and counterstained with methyl green. Controls included hybridizations with sense and unrelated probes.

### Peptide stimulation

The peptides OVA (SIINFEKL) or VSV (RGYVYQGL) were injected i.v. into OT-I *Tap1*<sup>-/-</sup> mice as described<sup>26</sup>. After 1h, the thymus was removed, RNA extracted, and subjected to real time RT-PCR.

### Statistical testing

Statistical differences were calculated using the mean difference hypothesis of Student's two tailed t-test assuming different variances and confidence level of 95%. Calculations performed using GraphPad Prism.S

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgements

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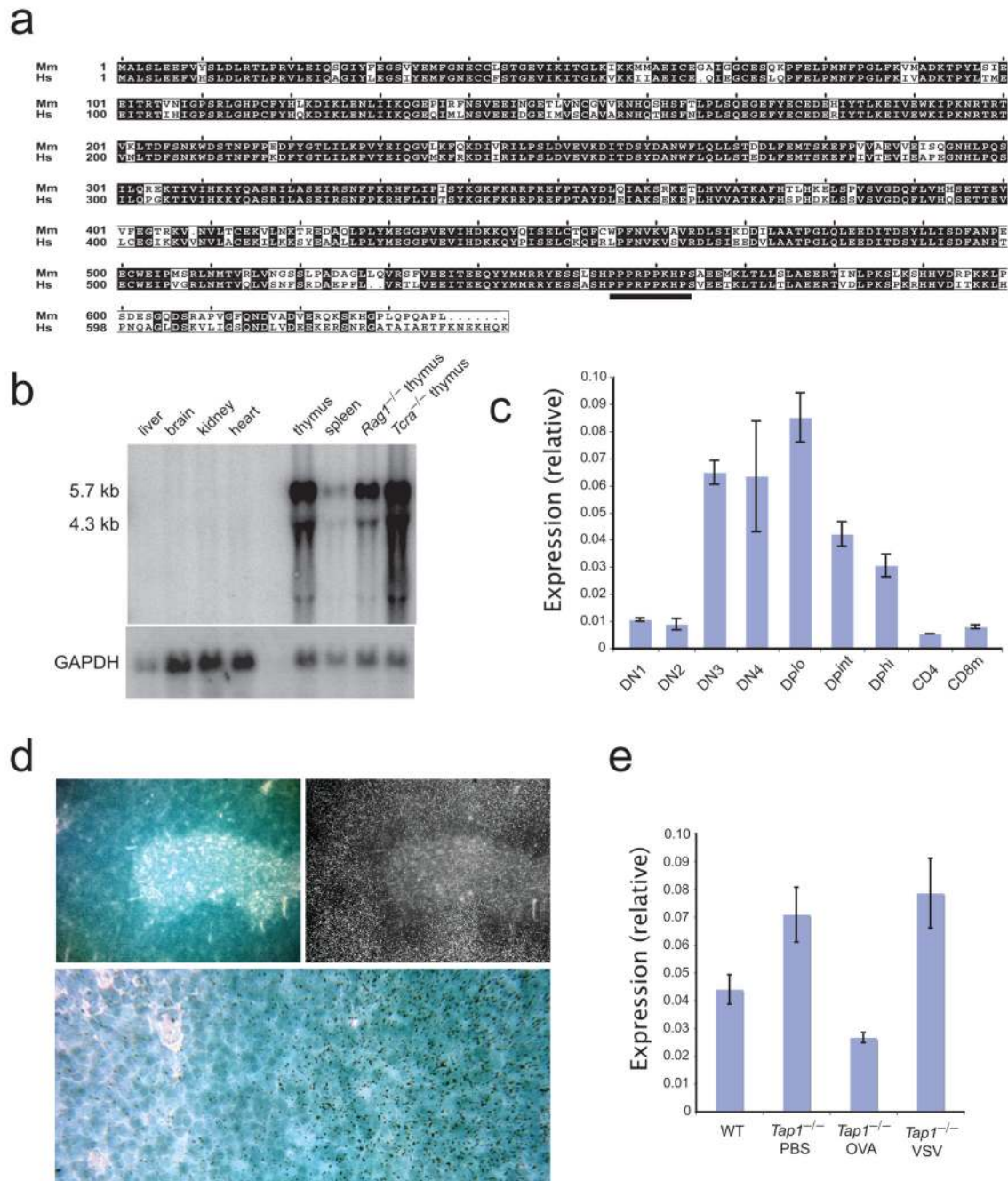
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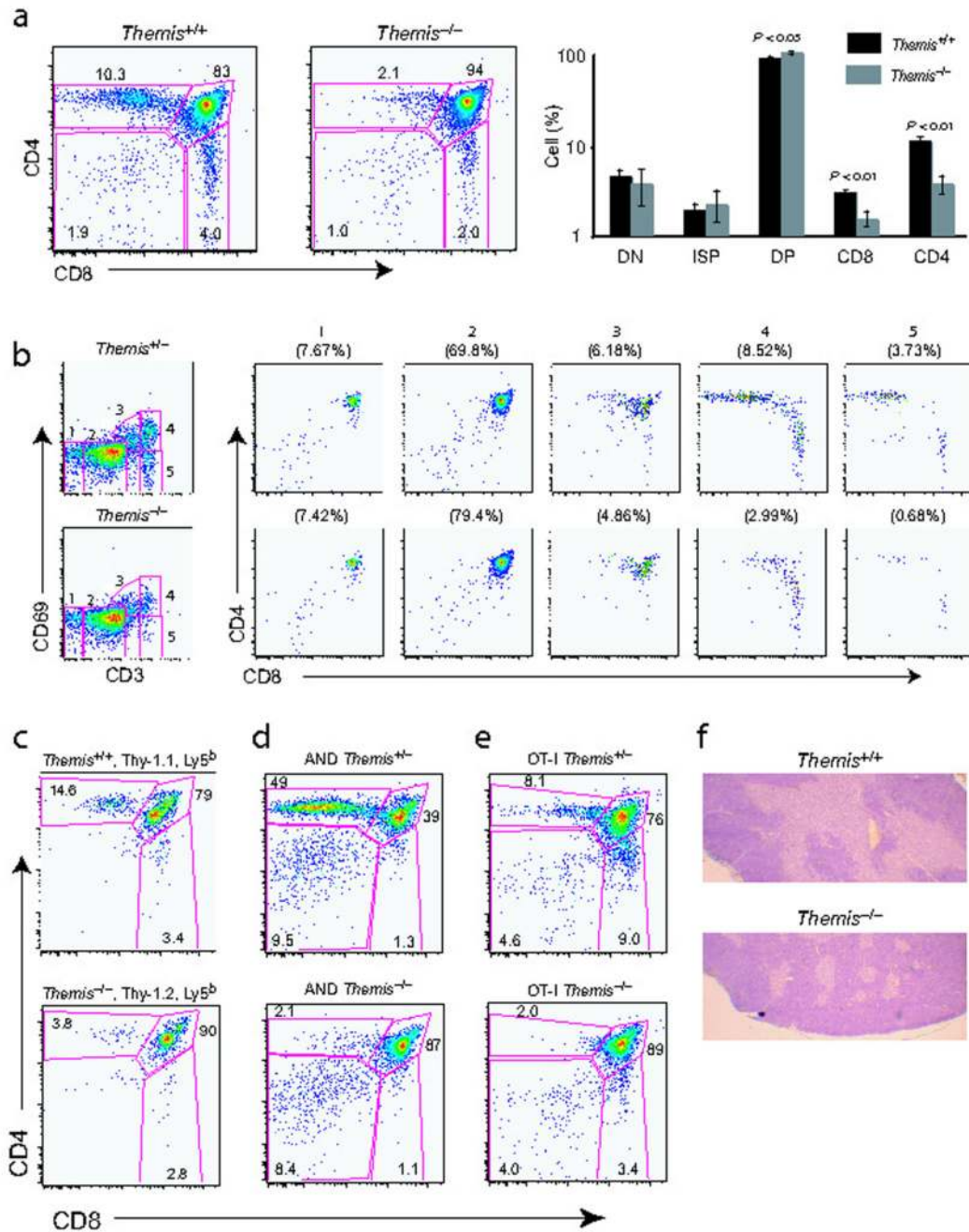
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**Figure 1.**

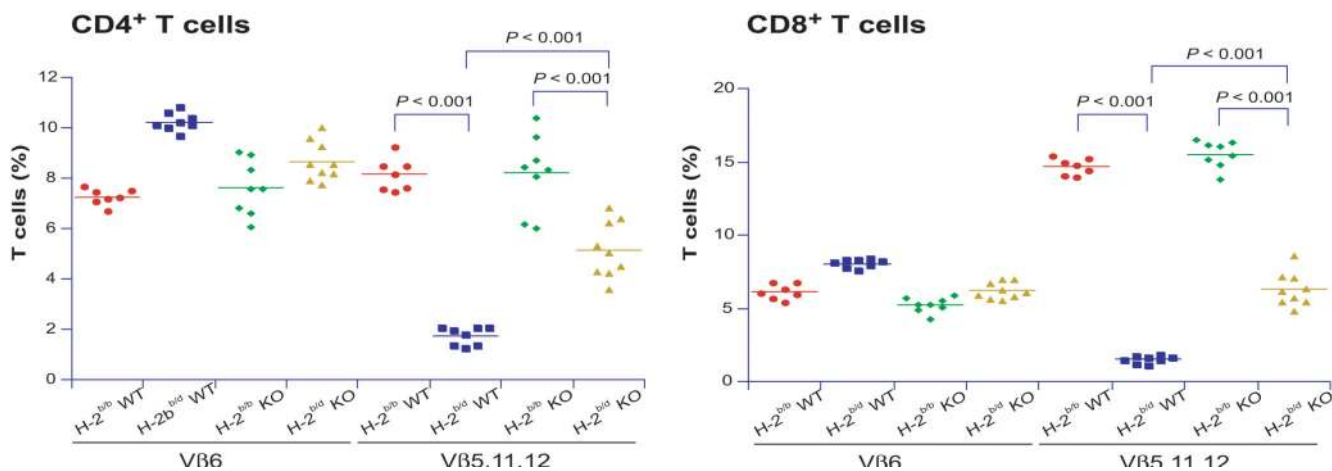
Themis structure, expression and localization in wild-type mice. **(a)** Alignment of predicted amino acid sequence of murine (Mm) and human (Hs) Themis proteins. Sequence alignments and comparisons were performed using Multalin46, and rendered using ESPript47. Polyproline region is underlined. Amino acid identity shown as white-on-black. **(b)** Northern blot of *Themis* RNA from different tissues. Representative of three independent experiments. **(c)** *Themis* mRNA expression analyzed by real-time RT-PCR of FACS-sorted subsets of B6 thymocytes. CD8m refers to mature CD8SP cells. Data shown relative to  $\beta$ -

actin expression. Representative of three experiments. Error bars denote s.d. **(d)** *In situ* hybridization of mouse thymus with *Themis* probe. Top left, brightfield image showing *Themis* expression as black dots. Original magnification  $\times 10$ . Top right, same image in darkfield showing *Themis* expression as white dots. Bottom, brightfield image of corticomedullary junction (cortex on right, medulla on left). Original magnification  $\times 40$ . Representative of 2 independent experiments, 3 mice each. **(e)** OT-I TCR tg *Tap1*<sup>-/-</sup> mice were injected i.v. with PBS, OVA or VSV peptide. Thymocytes were isolated and *Themis* expression analyzed by real-time RT-PCR. Note that OT-I tg *Tap1*<sup>-/-</sup> mice do not have any mature T cells or thymocytes specific for the injected peptide, so they would not be expected to undergo apoptosis due to cytokine release. FACS analysis did not show any increase in the percentage of dead cells in thymocytes from the treated mice, nor was there any change in the percentage of DP or SP subsets (data not shown). Representative of 4 experiments.

**Figure 2.**

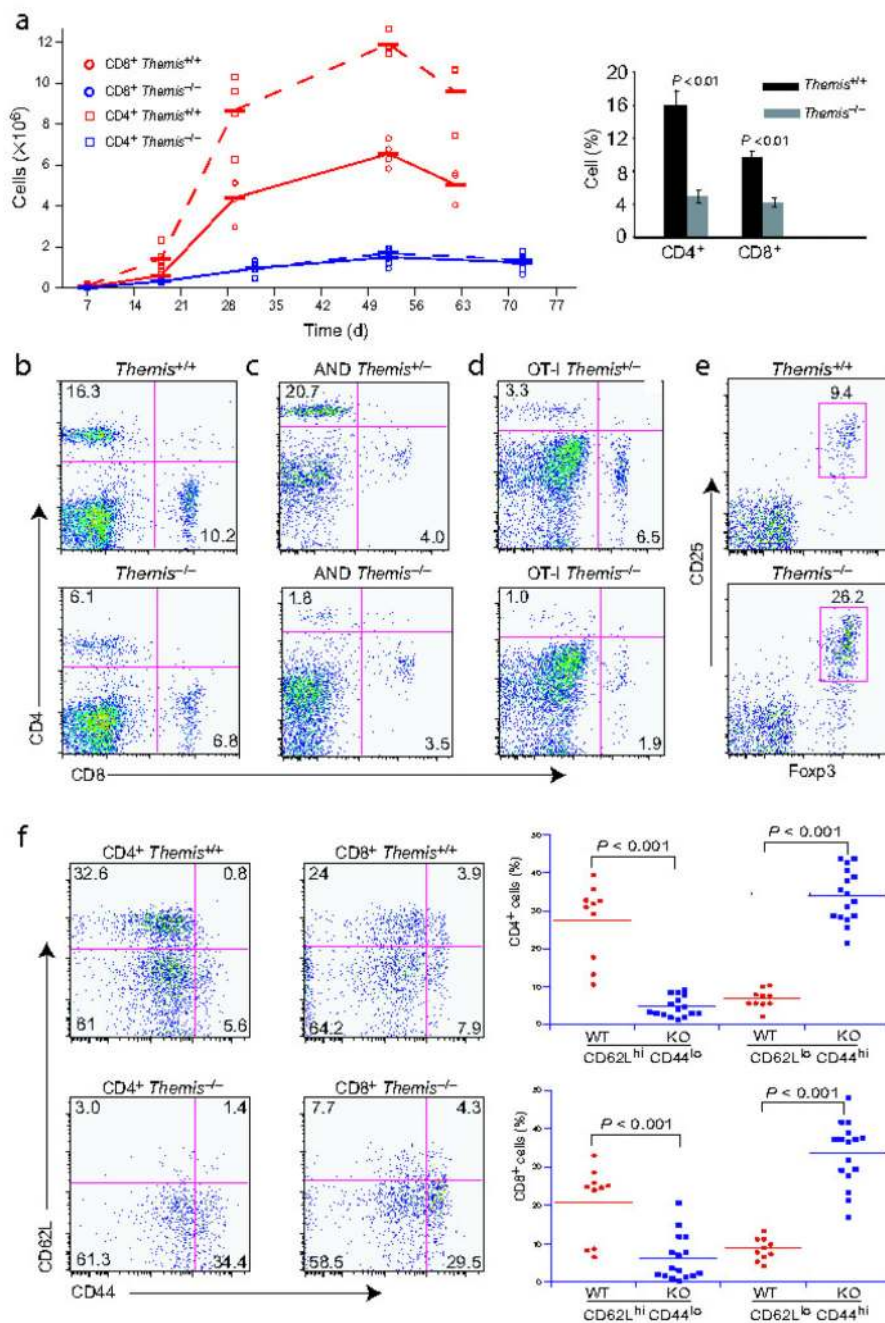
Defective positive selection in *Themis*<sup>-/-</sup> mice. **(a)** Thymocytes from *Themis*<sup>+/+</sup> and *Themis*<sup>-/-</sup> mice (intercross from line backcrossed  $\times 10$  to B6) were analyzed by FACS. Bar graph shows percentages of different thymocyte sub-populations from 7 mice analyzed in one experiment. Dot plots show representative staining. Results representative of  $>3$  experiments. **(b)** Differential surface expression of CD69 and CD3 was used to identify thymocyte populations of different maturity in *Themis*<sup>+/-</sup> (upper) and *Themis*<sup>-/-</sup> (lower) mice. Plots on the right are gated on subpopulations indicated in the left-most plot.

Percentage of thymocytes in each of the subpopulations shown above each panel. Representative of 2 mice each in 2 independent experiments. **(c)** Thymocyte sub-populations from irradiated Thy1.2Ly5<sup>a</sup> mice were reconstituted with a 1:1 mixture of BM from Thy1.2, Ly5<sup>b</sup> *Themis*<sup>-/-</sup> and Thy1.1, Ly5<sup>b</sup> *Themis*<sup>+/+</sup> mice. Donor populations were identified by Thy1.1 or Thy1.2 expression of Ly5<sup>b+</sup> cells. Two chimeric mice per group were analyzed in 3 separate experiments with similar results. **(d,e)** Thymocytes from *Themis*<sup>+/+</sup> and *Themis*<sup>-/-</sup> mice expressing the AND TCR27 **(d)** or the OT-I TCR24 **(e)** were analyzed by FACS. Significant differences ( $P < 0.0005$ , t-test) were found between the percentage of CD4 SP **(d)** and CD8 SP **(e)** cells in *Themis*<sup>+/+</sup> and *Themis*<sup>-/-</sup> thymi (data pooled from >3 separate experiments each); *Themis*<sup>+/+</sup> n=4 **(d)**, n=8 **(e)**; and *Themis*<sup>-/-</sup> n=6 **(d)**, n=12 **(e)**. **(f)** Thymus sections from *Themis*<sup>+/+</sup> and *Themis*<sup>-/-</sup> mice were stained with hematoxylin. Medulla shows lighter staining. Original magnification ×10. Representative of two experiments.

**Figure 3.**

Negative selection defect in *Themis*<sup>-/-</sup> mice. B6 mice (H-2<sup>b</sup>) bearing wild-type or mutated *Themis* alleles were backcrossed or not onto B10.D2 (H-2<sup>d</sup>) mice and the expression of indicated V $\beta$  elements in CD4 or CD8 peripheral blood T cells was analyzed by flow cytometry. V $\beta$ 5, V $\beta$ 11, and V $\beta$ 12 are deleted by the Mtv-8 and Mtv-9 superantigens expressed in B6 and B10.D2, but only when I-E is also expressed. Thus deletion occurs on H-2<sup>b/d</sup> but not H-2<sup>b/b</sup> backgrounds. V $\beta$ 6 is not deleted by these Mtv superantigens. Representative of three independent experiments.



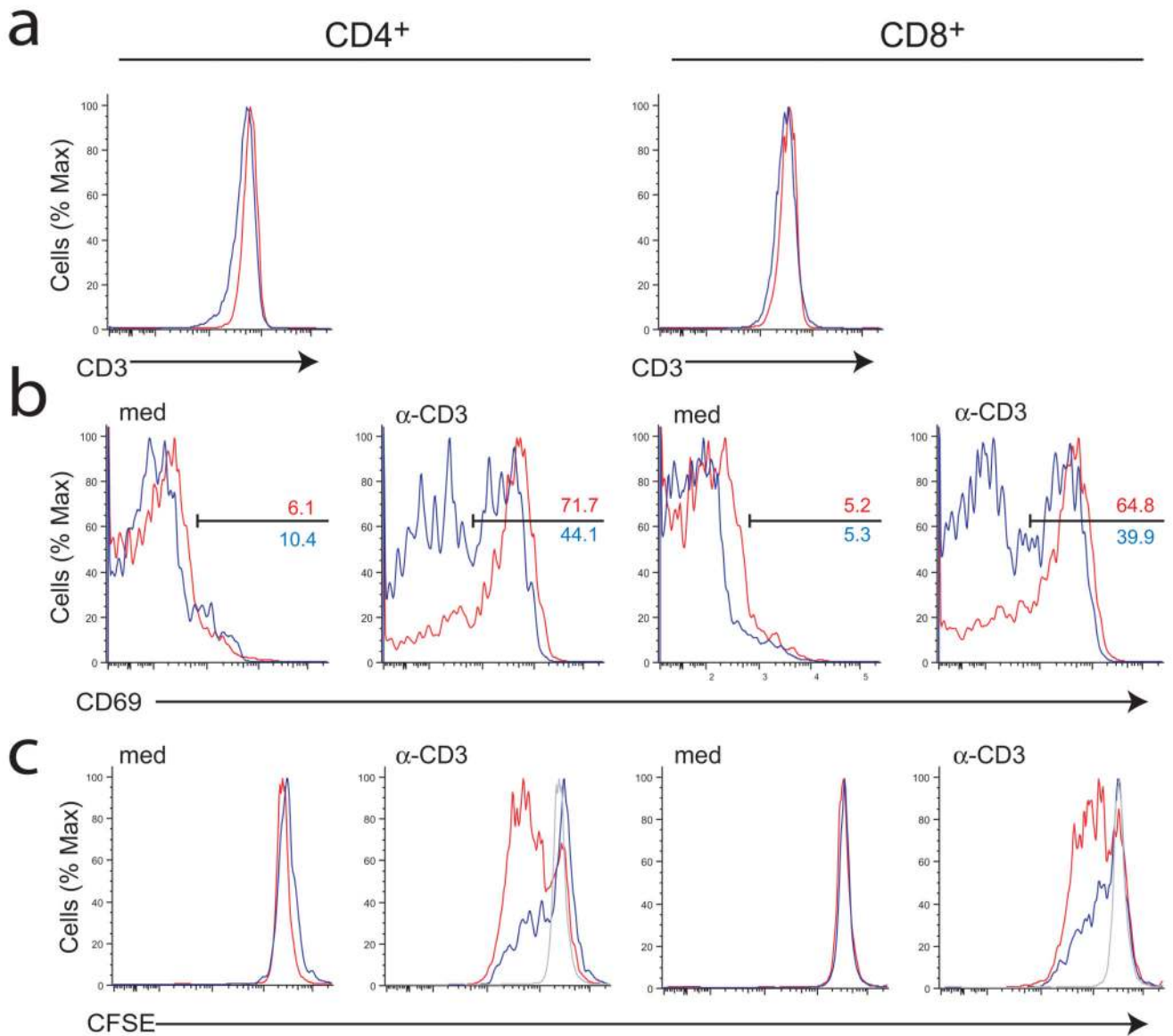


**Figure 4.**

Peripheral T cell phenotype in *Themis*<sup>-/-</sup> mice. **(a,b)** CD4 and CD8 T cells in the spleen of B6 *Themis*<sup>+/+</sup> and *Themis*<sup>-/-</sup> mice of indicated ages were quantified by flow cytometry. Bar graph shows average percentage of each population in seven 6 week old mice analyzed in one experiment. Dot plots show representative stainings. Data are representative of >3 experiments. **(c,d)** CD4 and CD8 expression on spleen cells from mice expressing AND **(c)** and **(d)** OT-I TCR transgenes. Differences between AND *Themis*<sup>+/+</sup> and *Themis*<sup>-/-</sup> CD4 T cell percentages ( $P < 0.0009$ ,  $n=4$  mice of each genotype), and *Themis*<sup>+/+</sup> and *Themis*<sup>-/-</sup> OT-

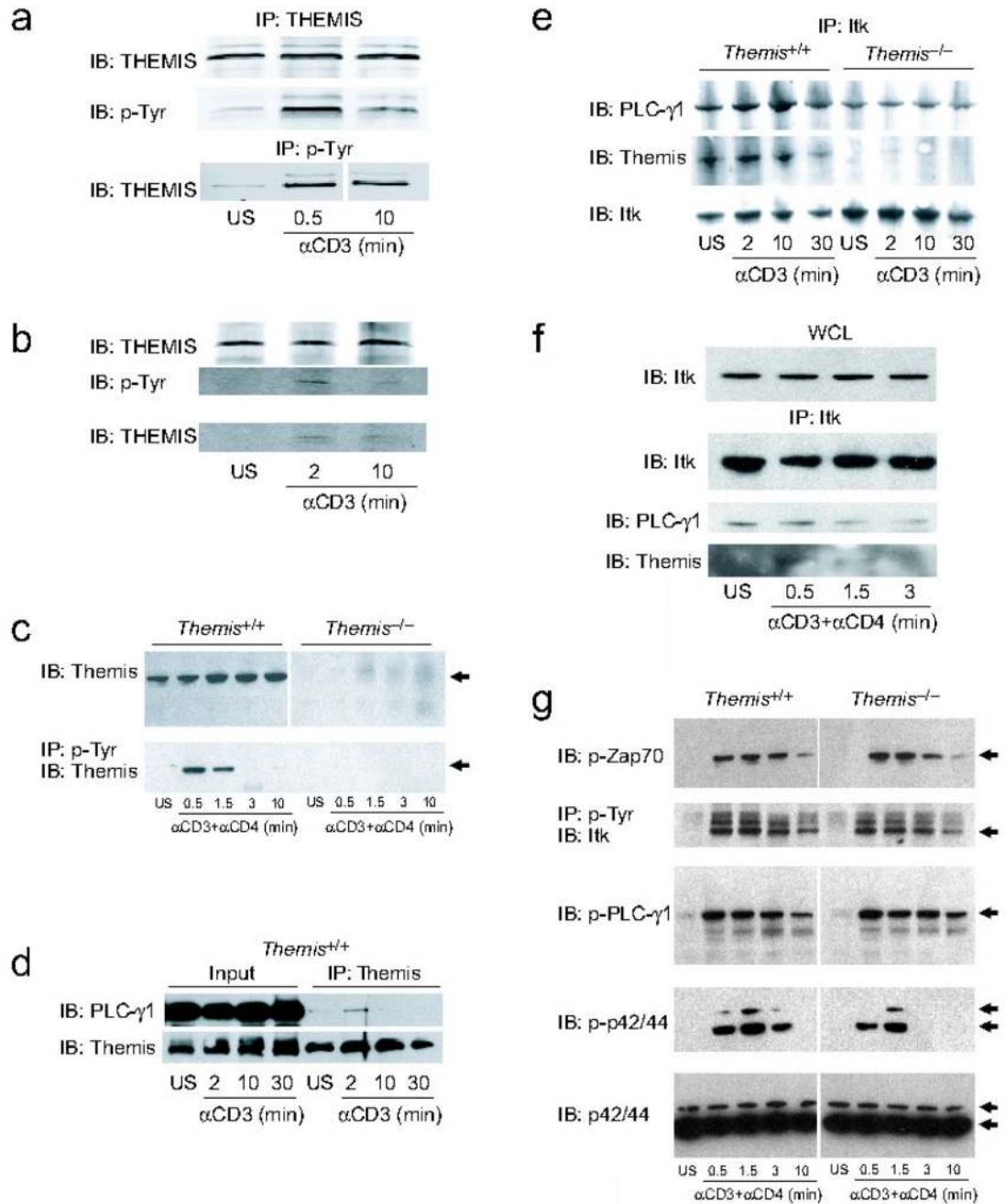


I CD8 T cell percentages ( $P < 0.003$ , *Themis*<sup>-/-</sup> n=7, *Themis*<sup>+/+</sup> n=5) were significant and representative of at least 3 individual experiments. (e) Increased percentage of CD25<sup>+</sup>Foxp3<sup>+</sup> cells in *Themis*<sup>-/-</sup> compared to *Themis*<sup>+/+</sup> splenocytes. Dot plots are gated on CD4<sup>+</sup> cells. ( $P = 2.8 \times 10^{-5}$ , n=4 mice of each genotype, representative of 2 independent experiments). (f) Expression of CD62L and CD44 on lymph node T cells from *Themis*<sup>+/+</sup> and *Themis*<sup>-/-</sup> mice. In graphs each dot represents an individual mouse. Dot plots show representative staining, and are gated on CD4<sup>+</sup> or CD8<sup>+</sup> cells as indicated. Representative of 3 independent experiments.



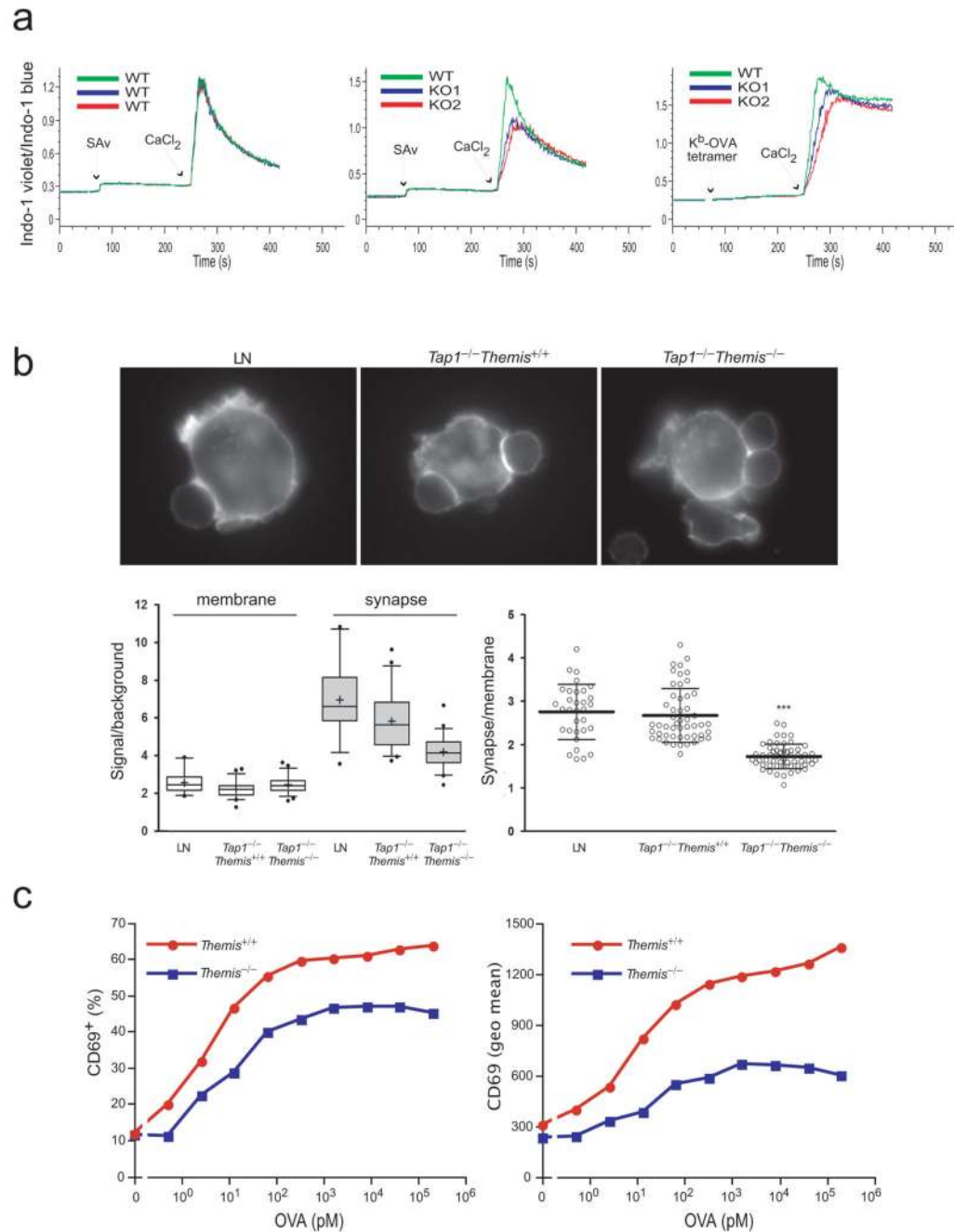
**Figure 5.**

Defective activation of *Themis*-deficient T cells. *Themis*<sup>-/-</sup> cells are shown in blue and *Themis*<sup>+/+</sup> cells in red. **(a)** CD3 expression on gated CD4<sup>+</sup> (CD25<sup>-</sup>) and CD8<sup>+</sup> spleen cells. **(b)** Spleen cells were activated by cross-linking with anti-CD3 for 5 hours, after which CD69 upregulation was analyzed on gated CD4<sup>+</sup> or CD8<sup>+</sup> cells. Med, unstimulated cells. **(c)** Spleen cells were labeled with CFSE and stimulated *in vitro* with anti-CD3 for 3 days. CD4<sup>+</sup> and CD8<sup>+</sup> splenocytes were gated and CFSE dilution was analyzed by flow cytometry. Each panel representative of at least 3 independent experiments.

**Figure 6.**

Themis is part of the TCR signal cascade. Jurkat **(a)** or fresh human PBL T cells **(b)** were stimulated with anti-CD3 for the indicated times (min, US is unstimulated), lysed and immunoprecipitated with anti-THEMIS serum or anti-p-Tyr mAb. Blots were probed with the same Abs. **(a)** and **(b)** are representative of five and two experiments, respectively. **(c)** Rested *Themis*<sup>+/+</sup> and *Themis*<sup>-/-</sup> thymocytes were stimulated with anti-CD3 plus anti-CD4, and whole cell lysate (WCL) or anti-p-Tyr immunoprecipitates were blotted and probed with anti-Themis. Representative of three experiments. **(d)** Freshly isolated B6 thymocytes were

activated with anti-CD3 and lysates were immunoprecipitated with anti-Themis. The blot was probed with anti-PLC- $\gamma$ 1 or anti-Themis. Representative of two experiments. **(e)** Freshly isolated *Themis*<sup>+/+</sup> or *Themis*<sup>-/-</sup> thymocytes were stimulated with anti-CD3, lysed and precipitated with anti-Itk, then blotted and probed with anti-PLC- $\gamma$ 1, anti-Themis and anti-Itk. In some experiments, mature thymocytes were depleted by MACS using anti-CD53, and cells were stimulated with anti-CD3 plus anti-CD4, with similar results. Representative of three experiments. **(f)** Rested B6 thymocytes were activated with anti-CD3 plus anti-CD4, lysed and immunoprecipitated with anti-Itk. Immunoprecipitate or WCL blots were probed with anti-PLC- $\gamma$ 1, anti-Themis and anti-Itk. Representative of two experiments. **(g)** Rested *Themis*<sup>+/+</sup> and *Themis*<sup>-/-</sup> thymocytes were stimulated with anti-CD3 plus anti-CD4, and WCL or anti-p-Tyr immunoprecipitates were blotted and probed with anti-p-ZAP70, anti-Itk, anti-p-PLC- $\gamma$ 1, and anti-p-p42/44 (Erk1/2) or anti-p42/44. Representative of three experiments.

**Figure 7.**

Signaling in *Themis*-deficient thymocytes. **(a)** Pre-selection DP thymocytes from *Themis*<sup>+/+</sup> or *Themis*<sup>-/-</sup> OT-I *Tap1*<sup>-/-</sup> mice were stimulated by crosslinking biotinylated anti-CD3 and -CD4 with streptavidin (SAv) (left and center) or with OVA-K<sup>b</sup> tetramer (right) in Ca<sup>2+</sup>-free medium. CaCl<sub>2</sub> was added later. Cells were surface-labeled with CFSE, Cy5 or nothing, mixed and assayed in one tube so that both *Themis*<sup>+/+</sup> or *Themis*<sup>-/-</sup> cells were tested at the same time in the same environment. Left; 3 samples of *Themis*<sup>+/+</sup> cells tested against each other, center and right; *Themis*<sup>+/+</sup> cells tested against cells from two different *Themis*<sup>-/-</sup>

mice. Representative of three independent experiments. **(b)** OT-I T cells or pre-selection DP thymocytes, *Themis*<sup>+/+</sup> or *Themis*<sup>-/-</sup>, were incubated with EL4 cells loaded with OVA peptide and stained to detect polymerized actin. Amount of f-actin in the immunological synapse versus the rest of the T cell membrane was calculated from imaging data and expressed in the left graph as the actin signal at the membrane within or outside of the synapse (boxes, s.e.m.; crossbar, median; plus sign, mean; bars, 5–95 percentile range; dots, data points outside this range). Right graph shows ratio of f-actin in the synapse to that in the non-synapse membrane. Each circle represents a single data point, bold lines show the mean, thinner lines show  $\pm$  s.e.m. \*\*\* $P < 0.05$ . Representative of two experiments. **(c)** Pre-selection DP thymocytes from OT-I *Tap1*<sup>-/-</sup>*Themis*<sup>+/+</sup> or *Themis*<sup>-/-</sup> mice were stimulated *in vitro* with OVA presented by EL4 cells and CD69 upregulation analyzed<sup>39,40</sup>. Representative of three experiments.