In vivo functional mapping of the conserved protein domains within murine Themis1

Ekaterina Zvezdova1, Jan Lee1, Dalal El-Khoury1, Valarie Barr2, Itoro Akpan2, Lawrence Samelson2 and Paul E Love1

Thymocyte development requires the coordinated input of signals that originate from numerous cell surface molecules. Although the majority of thymocyte signal-initiating receptors are lineage-specific, most trigger 'ubiquitous' downstream signaling pathways. T-lineage-specific receptors are coupled to these signaling pathways by lymphocyte-restricted adapter molecules. We and others recently identified a new putative adapter protein, Themis1, whose expression is largely restricted to the T lineage. Mice lacking Themis1 exhibit a severe block in thymocyte development and a striking paucity of mature T cells revealing a critical role for Themis1 in T-cell maturation. Themis1 orthologs contain three conserved domains: a proline-rich region (PRR) that binds to the ubiquitous cytosolic adapter Grb2, a nuclear localization sequence (NLS), and two copies of a novel cysteine-containing globular (CABIT) domain. In the present study, we evaluated the functional importance of each of these motifs by retroviral reconstitution of Themis1⁻/⁻ progenitor cells. The results demonstrate an essential requirement for the PRR and NLS motifs but not the conserved CABIT cysteines for Themis1 function.

Immunology and Cell Biology (2014) 92, 721–728; doi:10.1038/icb.2014.43; published online 17 June 2014

Themis1 is the founding member of a new gene family with orthologs in almost all animal species. In mammals, three related family members, Themis1, Themis2 and Themis3, have been detected that exhibit highly tissue-specific expression in the T-cell (Themis1), B-cell, myeloid and dendritic cell (Themis2) and intestinal epithelial cell (Themis3) lineages.1 Analysis of induced loss-of-function Themis1 mutants by several independent groups has revealed a critical function for Themis1 in later stages of T-cell development, including positive selection and the generation of mature T cells.1,2 Specifically, Themis1⁻/⁻ mice show a severe block at the CD4⁺CD8⁺ (double positive, DP) to CD4⁺CD8⁻ (CD4-single positive, CD4-SP) or CD4⁻CD8⁺ (CD8-single positive, CD8-SP) stages that results in a marked reduction in numbers of CD4-SP and CD8-SP thymocytes and T cells.1,2 To date, only Themis1 has been inactivated in the mouse germline, and it therefore remains unknown whether Themis2 and Themis3 have similarly important roles in the cell lineages in which they are expressed.

Themis family members do not contain any known catalytic domains. However, sequence alignment of Themis1 and Themis2 identifies three highly conserved regions of potential functional relevance: (1) a carboxy-terminal proline-rich region (PRR) (PPPRPPKPKP) that matches a Class II SH3 recognition motif (PxxPx⁺); (2) a bipartite nuclear localization sequence (NLS) (PKR-X12-KRRPR), and (3) two copies of a newly described cysteine-containing All-Beta in Themis (CABIT) domain (ΦXCX7-26ΦXLPΦX3GXF with Φ = any amino acid and Φ = any hydrophobic residue).1,2 Outside of these three domains, Themis1 and Themis2 are poorly conserved.3 Compelling evidence that one or all of the conserved regions are important for function is the recent finding that a Themis2 transgene is capable of restoring normal T-cell development in mice lacking Themis1.4

In this study, we investigated the importance of each of the three conserved domains for Themis1 function by generating retroviral constructs that encode Themis1 proteins in which one domain has been mutated and testing the ability of these constructs to rescue T-cell development in Themis1⁻/⁻ thymocytes. Our results demonstrate that whereas retention of key cysteine residues within the CABIT domain are not required for function, the PRR and NLS motifs are each essential for Themis1 activity. Our data also suggest that interaction of Themis1 with the adapter Grb2, and possibly Themis1-mediated nuclear localization of Grb2, may be critical for Themis1 function and for normal T-cell maturation.

RESULTS
Generation and in vitro characterization of Themis1 mutants
Vertebrate Themis1 orthologs contain three highly conserved motifs that represent potential functional domains: (1) a carboxy-terminal PRR, (2) a bipartite NLS, and (3) two CABIT domains.1,2 To investigate the importance of each of these motifs for Themis1 function, we generated FLAG-epitope tagged Themis1 cDNAs encoding either wild-type (WT) Themis1 or proteins in which one domain has been mutated.

--

1Section on Cellular and Developmental Biology, Program on Genomics of Differentiation, Eunice Kennedy Shriver, National Institute of Child Health and Human Development, Bethesda, MD, USA and 2Laboratory of Cellular and Molecular Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA.

Correspondence: Dr PE Love, Section on Cellular and Developmental Biology, Program on Genomics of Differentiation, Eunice Kennedy Shriver, National Institute of Child Health and Human Development, National Institute of Health, Room 2B-210, Building 6B, Bethesda, MD 20892, USA.

E-mail: lovep@mail.nih.gov

Received 22 January 2014; revised 20 April 2014; accepted 11 May 2014; published online 17 June 2014
versions of Themis1 included Themis-ΔPRR in which the PPPRPP amino acids of the PRR were deleted, Themis-ΔNLS1 in which the PKR amino acids of the N-terminal domain of the bipartite NLS were deleted and Themis-ΔCys153/413 (Themis-ΔCys) in which both of the conserved CABIT domain cysteine residues was changed to alanine (Figure 1a).

Transfection of the HEK 293T cell line with plasmids encoding WT or mutant Themis1 proteins confirmed expression of FLAG epitope-tagged proteins of the predicted size (Figure 1b). We and others have shown that, in thymocytes, Themis1 binds constitutively to the ubiquitous adapter protein Grb2.1–3,7 This association is mediated by the Themis1 PRR, which can bind to either the N-terminal SH3 domain or the C-terminal SH3 domain of Grb2. As expected, deletion of the conserved PRR motif resulted in complete loss of Grb2-binding potential (Figure 1b). In contrast, mutation of the conserved cysteines within the two CABIT domains did not affect Themis1:Grb2 association. Grb2 association with Themis-ΔNLS1 was reduced in comparison to WT Themis1 but mutation of the NLS did not abrogate Grb2 binding (Figure 1b and data not shown).

We have also previously demonstrated that Themis1 protein is detectable in both the nucleus and cytoplasm in thymocytes.1,6 In addition, we found that nuclear Grb2 is markedly reduced in Themis1−/− thymocytes, suggesting that Themis1 may be important for nuclear translocation and/or retention of Grb2 in thymocytes (Figure 1c). Consequently, we evaluated the importance of each of the three conserved domains for nuclear localization of Themis1. Themis-ΔNLS1 was undetectable in the nuclear subcellular fraction, indicating that an intact bipartite NLS sequence is necessary for Themis1 nuclear translocation and/or retention (Figure 1d). In contrast, the relative cytoplasmic/nuclear distribution of Themis1 protein was similar in

![Diagram of protein structure and sequences](image-url)

**Figure 1** Generation and characterization of Themis1 mutants lacking individual conserved domains. (a) Top, schematic of Themis1 protein structure showing the location of the two novel globular CABIT domains (CABIT-1 and CABIT-2) each containing a conserved cysteine (Cys) residue, the bipartite NLS within the two CABIT domains did not affect Themis1:Grb2 association. Grb2 association with Themis-ΔNLS1 was reduced in comparison to WT Themis1 but mutation of the NLS did not abrogate Grb2 binding (Figure 1b and data not shown).

We have also previously demonstrated that Themis1 protein is detectable in both the nucleus and cytoplasm in thymocytes.1,6 In addition, we found that nuclear Grb2 is markedly reduced in Themis1−/− thymocytes, suggesting that Themis1 may be important for nuclear translocation and/or retention of Grb2 in thymocytes (Figure 1c). Consequently, we evaluated the importance of each of the three conserved domains for nuclear localization of Themis1. Themis-ΔNLS1 was undetectable in the nuclear subcellular fraction, indicating that an intact bipartite NLS sequence is necessary for Themis1 nuclear translocation and/or retention (Figure 1d). In contrast, the relative cytoplasmic/nuclear distribution of Themis1 protein was similar in

![Diagram of protein structure and sequences](image-url)

**Figure 1** Generation and characterization of Themis1 mutants lacking individual conserved domains. (a) Top, schematic of Themis1 protein structure showing the location of the two novel globular CABIT domains (CABIT-1 and CABIT-2) each containing a conserved cysteine (Cys) residue, the bipartite NLS within the two CABIT domains did not affect Themis1:Grb2 association. Grb2 association with Themis-ΔNLS1 was reduced in comparison to WT Themis1 but mutation of the NLS did not abrogate Grb2 binding (Figure 1b and data not shown).

We have also previously demonstrated that Themis1 protein is detectable in both the nucleus and cytoplasm in thymocytes.1,6 In addition, we found that nuclear Grb2 is markedly reduced in Themis1−/− thymocytes, suggesting that Themis1 may be important for nuclear translocation and/or retention of Grb2 in thymocytes (Figure 1c). Consequently, we evaluated the importance of each of the three conserved domains for nuclear localization of Themis1. Themis-ΔNLS1 was undetectable in the nuclear subcellular fraction, indicating that an intact bipartite NLS sequence is necessary for Themis1 nuclear translocation and/or retention (Figure 1d). In contrast, the relative cytoplasmic/nuclear distribution of Themis1 protein was similar in
WT Themis1, Themis-ΔPRR and Themis-ΔCys-transfected cells, indicating that the conserved CABIT cysteines and PRR sequence are not essential for Themis1 nuclear localization/retention (Figure 1d). Moreover, the observation that nuclear localization of Themis-ΔPRR is unimpaired demonstrates that Themis1 nuclear transport and/or retention is not dependent upon Grb2 association.

To directly visualize Themis1 localization in T cells, we next transfected the human Jurkat T cell line with Myc epitope tagged WT, ΔPRR or ΔNLS1 versions of Themis1. Examination of Themis1 localization in transfected Jurkat cells confirmed a requirement for the NLS domain, but not the PRR domain, for nuclear localization of Themis1 (Figure 2). We also examined cellular localization of endogenous Grb2 in transfected cells using anti-Grb2 antibody. Overall, there was no significant effect on the cellular distribution of Grb2 even in cells expressing Themis-ANLS1 (Figure 2 and data not shown). However, it is important to note that these cells also contain endogenous WT Themis1, which is capable of binding to Grb2.

In vivo testing of Themis1 mutants by retroviral genetic reconstitution

To evaluate the importance of each of the Themis1 conserved domains for in vivo function, we generated retroviral constructs that encoded WT Themis1, Themis-ΔPRR, Themis-ANLS1 or Themis-ΔCys. Lineage-negative bone marrow cells from C57BL/6 (CD45.2⁺) Themis⁻/⁻ mice were infected with retrovirus and then injected intravenously into lethally irradiated congenic CD45.1⁺ mice. Eight weeks later, thymocytes and T cells from peripheral bone marrow cells were assayed by flow cytometry. As the retroviral vectors contained tandem Themis1-IRE5-EGFP (enhanced green fluorescent protein) cassettes, expression of retroviral-encoded genes could be identified by gating on CD45.2⁺ EGFP⁺ cells. Intracellular staining of CD45.2⁺ EGFP⁺ thymocytes from bone marrow chimeric mice with antibody against Themis1 demonstrated similar expression of WT Themis1, Themis-ΔPRR, Themis-ANLS1 or Themis-ΔCys retroviral-infected cells (Figure 3a). Having established equivalent expression of the retroviral-encoded Themis1 proteins, we next validated the experimental system by comparing mice reconstituted with Themis1⁻/⁻ bone marrow cells that had been infected with either empty retrovirus (Empty) or retrovirus encoding WT Themis1. As expected, the phenotype of thymocytes derived from Empty retrovirus-infected Themis1⁻/⁻ bone marrow cells closely resembled that of Themis1⁻/⁻ mice exhibiting a profound block at the DP to CD4-SP or CD8-SP stages (Figure 3b). Moreover, the percentage of TCR⁺ thymocytes, which include both late-stage positively selected DP thymocytes and SP thymocytes, as well as CD24⁺ thymocytes which are composed primarily of mature CD4-SP and CD8-SP thymocytes, was strongly reduced in thymocytes derived from Empty retrovirus-infected Themis1⁻/⁻ progenitors (Figure 3b). Also similar to Themis1⁻/⁻ mice, the percentage of peripheral lymph node CD45.2⁺ EGFP⁺ CD4-SP and CD8-SP T cells was dramatically reduced in these mice (Figures 4a and b). In contrast, based on the same criteria, the phenotype of thymocytes and peripheral T cells derived from WT Themis1 retrovirus-infected Themis1⁻/⁻ bone marrow cells exhibited an essentially normal phenotype (as compared with Themis1⁺/⁺ mice), indicating that genetic reconstitution of WT Themis1 can completely ‘rescue’ the developmental defects in Themis1⁻/⁻ mice (Figures 3 and 4).

Themis-ΔCys was capable of restoring thymocyte development when expressed in Themis1⁻/⁻ progenitor cells. The percentage of EGFP⁺ CD4-SP and CD8-SP thymocytes, and more importantly, mature CD24⁺ CD4-SP and CD8-SP thymocytes was significantly increased relative to mice reconstituted with Empty vector-infected bone marrow cells and relative to non-retroviral-infected host Themis1⁻/⁻ thymocytes, though the extent of rescue appeared to be slightly less than observed with WT Themis1 (Figures 3b and c). However, the percentage of CD45.2⁺ EGFP⁺ CD4-SP and CD8-SP peripheral T cells was similar to that observed in mice where Themis1⁻/⁻ progenitors were infected with WT Themis1 (Figures 4a and b). In contrast to Themis-ΔCys, Themis-ΔPRR and Themis-ANLS1 failed to rescue thymocyte development when introduced into Themis1⁻/⁻ progenitors. Neither protein was capable of promoting the maturation of CD4-SP or CD8-SP thymocytes (Figures 3b and c) and the percentage of peripheral EGFP⁺ T cells was not significantly different than in mice that had been reconstituted with Empty retrovirus (Figures 4a and b).

To determine whether Themis-ΔPRR, Themis-ΔNLS1 or Themis-ΔCys effect thymocyte development in the presence of WT Themis1, we infected bone marrow progenitors from WT (B6-CD45.1) mice with retrovirus encoding WT Themis1 or the three Themis1 mutants. Interestingly, expression of either WT Themis1 or Themis-ΔCys resulted in a consistent though not statistically significant increase in CD4-SP and CD8-SP T thymocytes and peripheral CD4-SP and CD8-SP T cells (Figures 5 and 6). These results are consistent with data obtained with Themis1 transgenic mice (data not shown) and suggest that T-cell maturation can be enhanced by augmentation of Themis1 expression by either WT Themis1 or Themis-ΔCys. Expression of Themis-ΔPPR or Themis-ΔNLS1 in WT bone marrow progenitors did not appreciably enhance or diminish T-cell maturation, indicating that neither protein exerted dominant-negative effect on thymocyte development in the presence of WT Themis1 (Figures 5 and 6).

DISCUSSION

The current results identify a critical role for the PRR and NLS domains of Themis1 for its function in regulating T-cell maturation. The observation that the PRR domain, which mediates Grb2 binding,
is required for Themis1 activity is consistent with recently published data demonstrating that Grb2 association is important for recruitment of Themis1 to the scaffolding adapter LAT in thymocytes. The PRR domain may also be required for the interaction of Themis1 with other cytoplasmic effector proteins such as the tyrosine phosphatase SHP-1 that are potential targets of Themis1 regulatory activity.

Notably, our results demonstrate a role for the NLS, and by extension, nuclear localization for Themis1 activity. Previous studies have shown that Grb2 can be detected in the nucleus as well as in the cytoplasm. In conjunction with our finding that nuclear Grb2 is reduced in Themis1−/− thymocytes, this raises the possibility that an important function of Themis1 may be to regulate transport of Grb2.

Figure 3 Importance of conserved Themis1 protein domains in thymocyte development. Lethally irradiated C57BL6/J (CD45.1+) mice were reconstituted with lineage-depleted Themis1−/− bone marrow cells (CD45.2+) that had been infected with a bicistronic EGFP-expressing retroviral vector (Empty vector) or the same vector encoding either wild-type Themis1 cDNA (WT) or mutant Themis1 proteins: Themis-ΔPRR (PRR), Themis-ΔNLS (NLS1) or Themis-ΔCys (Cys1/2). Eight weeks postreconstitution, thymocytes and splenocytes of bone marrow chimeras were analyzed by flow cytometry. (a) Intracellular staining for Themis1 in CD45.2+ EGFP+ CD4+CD8− (DP) thymocytes from bone marrow chimeric mice. Solid histograms represent Themis1 expression in EGFP-DP thymocytes. Unfilled histograms represent expression of Themis1 in EGFP+ DP thymocytes, expressing the indicated Themis1 proteins. (b) Representative flow cytometry analysis of CD45.2+ EGFP+ thymocytes from bone marrow chimeras. Upper plots show CD4 versus CD8 profiles, middle histograms show the percentage of TCRβ+ cells. Bottom plots show the percentage of mature CD45.2+ EGFP+ TCRβhiCD24lo thymocytes. Plots in right column show Themis1−/− thymocytes infected with empty retroviral vector for reference. (c) Percentage of mature (TCRβhiCD24lo) CD45.2+ EGFP+ CD4-SP and CD8-SP thymocytes in the indicated bone marrow chimeras (n=6 each). *P<0.05, N.S., not significant (two-tailed T-test, unequal variance) All comparisons are to vector only. A full color version of this figure is available at the *Immunology and Cell Biology* journal online.
and perhaps other Grb2-associated proteins such as the guanine nucleotide exchange factor Vav112 to the nucleus. We did not observe a defect in T-cell development in mice overexpressing Themis1-DNLS1. The absence of a discernable dominant-negative effect of Themis1-DNLS1 suggests that either Grb2 nuclear localization may not be essential for thymocyte maturation or that overexpression of Themis1-DNLS1 may not affect Grb2 nuclear translocation in thymocytes that also express WT Themis1.

Based on the high degree of conservation of the CABIT domain, which extends from mammals to cnidarians, it was proposed that the CABIT cysteine residues may be important for catalytic activity, possibly involving ubiquitination or thiol redox reactions.\(^2\) Our finding that the conserved cysteine residues are not essential for Themis1 activity argues against such a role, or alternatively, that the function performed by the CABIT domains is not essential for T-cell development. It is also possible that the conserved cysteine residues are not absolutely required for CABIT domain activity. Further in-depth analysis of the PRR, NLS and CABIT domains, including complementation studies, should provide valuable additional insight into Themis1 function and its precise role in T-cell signaling and thymocyte maturation.

**METHODS**

**Mice**

Themis1\(^{-/-}\) mice have been described previously.\(^1\) CD45.1 (B6.SJL-PtprcaPepcb/BoyJ) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Animal experiments were approved by the Animal Care and Use Committee of the National Institute of Child Health and Human Development, NIH.

**Cells and plasmids**

293T cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, penicillin, streptomycin and L-glutamine. WT (E6.1) Jurkat T cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics. Transient transfections were performed using the Amaxa electroporation system kit T (Amaxa Biosystems, Gaithersburg, MD, USA). For Myc-tagged Themis1 constructs, cDNA of Themis1 was amplified from thymocytes and cloned into pCDNA3.1 (Life Technologies, Grand Island, NY, USA). This plasmid was used as a template to delete the PRR region of Themis1 (RxPXXP), the N-terminal (PKR) part of the NLS, or to generate Cys to Ala amino-acid mutations in the CABIT1 and CABIT2 domains of Themis1. Mutagenesis reactions were performed using the GeneTailer mutagenesis kit from Invitrogen (Grand Island, NY, USA). Mutations were verified by sequencing. pMYs-IresEGFP

---

**Figure 4** (a) Representative flow cytometry analysis of CD45.2\(^+\) EGFP\(^+\) splenocytes from bone marrow chimeras shown in Figure 3. Upper plots show CD4 versus CD8 profiles, lower histograms show the percentage of TCR\(\beta\)\(^+\) T cells within the CD45.2\(^+\) EGFP\(^+\) population. Plots in right column show Themis1\(^{-/-}\) splenocytes infected with empty retroviral vector for reference. (b) Percentage of CD45.2\(^+\) EGFP\(^+\) CD4-SP and CD8-SP splenocytes in the indicated bone marrow chimeras (\(n=6\) each). *\(P\leq0.05\), **\(P\leq0.01\), N.S., not significant (two-tailed T-test, unequal variance). All comparisons are to vector only. A full color version of this figure is available at the Immunology and Cell Biology journal online.
retroviral plasmids (Cell Biolabs, San Diego, CA, USA) were used for expression of WT or mutant Themis1 proteins in vivo.

Retroviral transduction of bone marrow

Themis1<sup>+/−</sup> or Themis1<sup>−/−</sup> mice were treated with 5-fluorouracil (3 mg per mice) for 5 days. Bone marrow cells from treated mice were incubated with stem cell factor (100 ng ml<sup>−1</sup>) and thrombopoietin (50 ng ml<sup>−1</sup>) in Iscove’s Modified Dulbecco’s medium supplemented with 15% fetal bovine serum, Pen/Strep/Glut, 2-ME and nonessential amino acids in 24-well plates at a concentration of 2<sup>10<sup>6</sup> cells per well. Twenty-four hours later, cells were placed on retronectin (Takara Bio, Shiga, Japan) precoated 24-well plates with viral supernatant containing Polybrene (8 μg ml<sup>−1</sup>; Sigma, St Louis, MO, USA) and centrifuged at room temperature for 1 h at 1000 g. Cells were washed once with Iscove’s Modified Dulbecco’s media and placed in the culture with cytokines. A second round of infection was performed 1 day after. 3–5 x 10<sup>5</sup> cells were intravenously injected into lethally (950 rad) irradiated CD45.1 recipients. For virus generation, retroviral vectors (i.e., empty vector, WT-Themis1 vector or mutated Themis1 vector) were transfected into Plat-E packaging cells using the polyjet reagent (Signagen, Rockville, MD, USA). Viral supernatants were collected 48 h after transfection and stored at +4 °C.

The infection efficiency of bone marrow cells was determined by analyzing the percentage of GFP<sup>+</sup> cells by flow cytometry.

Antibodies and reagents

Sources for antibodies and reagents used in this study include: anti-laminB (M-20), anti-PLCγ (SC-81), obtained from Santa Cruz Biotechnology (San Diego, CA, USA), and anti-Grb2, obtained from BD Pharmingen (San Jose, CA, USA); anti-Themis1 rabbit antibodies were previously described.<sup>1</sup>

Subcellular fractionation

In all, 2 x 10<sup>6</sup> 293T cells were incubated in 120 μl of hypotonic buffer (HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) 100 mm, KCl 10 mm, EDTA 1 mm, Na3VO4 2 mm, protease inhibitor tablet (Roche, Nutley, NJ, USA)) for 20 min on ice. After incubation, 1.2 μl of 10% NP40 was added.

---

**Figure 5** Effect of Themis1 or Themis1 mutant protein expression on Themis<sup>+/−</sup> thymocyte development. Lethally irradiated C57BL6/J (CD45.1<sup>+</sup>) mice were reconstituted with lineage-depleted Themis1<sup>+/−</sup> bone marrow cells (CD45.2<sup>+</sup>) that had been infected with a bicistronic EGFP-expressing retroviral vector (Empty vector) or the same vector encoding either wild-type Themis1 CDNA (WT) or mutant Themis1 proteins: Themis<sup>−APRR</sup> (PRR), Themis<sup>−ANLS</sup> (NLS1) or Themis<sup>−ACys</sup> (Cys1/2). Eight weeks postreconstitution, thymocytes and splenocytes of bone marrow chimeras were analyzed by flow cytometry. (a) Representative flow cytometry analysis of CD45.2<sup>+</sup> EGFP<sup>+</sup> thymocytes from bone marrow chimeras. Upper plots show CD4 versus CD8 profiles, middle histograms show the percentage of TCRβ<sup>+</sup> cells. Bottom plots show the percentage of mature CD45.2<sup>+</sup> EGFP<sup>+</sup> TCRβ<sup>+</sup>CD24<sup>lo</sup> thymocytes. Plots in the left column show Themis1<sup>+/−</sup> thymocytes infected with empty retroviral vector for reference. (b) Percentage of mature (TCRβ<sup>+</sup>CD24<sup>lo</sup>) CD45.2<sup>+</sup> EGFP<sup>+</sup> CD4<sup>-</sup>SP and CD8<sup>-</sup>SP thymocytes in the indicated bone marrow chimeras (n=6 each). N.S., not significant (two-tailed T-test, unequal variance). All comparisons are to vector only. A full color version of this figure is available at the *Immunology and Cell Biology* journal online.
Lysates were mixed vigorously and centrifuged at 3000 r.p.m. for 5 min. Supernatants, which contained plasma membrane and cytosol, were collected. Pellets were washed with hypotonic buffer and incubated with 50 nM nuclear lysis buffer (Tris–HCL 100 mM, NaCl 300 mM, EDTA 1 mM, Na3VO4 2 mM, NP-40 1%, sodium dodecyl sulfate 0.1%, protease inhibitor tablet) for 10 min on ice. Lysates were centrifuged at 14 000 r.p.m. for 10 min. Supernatants, which contain nuclear extract, were collected.

**Intracellular staining and flow cytometry**

Thymocytes and spleen cells (1.6 × 10⁶ ml⁻¹) were surface stained with the following antibodies: CD4(RM4.5), CD8 (53–6.7), CD69 (H1.2F3), CD24 (30-F1), CD45.2 (104), CD5 (53–7.3), TCRβ (H57) obtained from eBioscience (San Diego, CA, USA). For intracellular staining, cells were fixed in 2% paraformaldehyde, permeabilized in 0.75% Triton-phosphate-buffered saline buffer and stained using rabbit anti-Themis1 followed by goat anti-rabbit Alexa647 antibody (Invitrogen). Acquisition was performed on a BD Biosciences LSR II (San Jose, CA, USA), and analysis was performed with FACS Diva software (BD Bioscience).

**In vitro binding experiments and immunoprecipitations**

In all, 2 × 10⁶ 293T cells were lysed in Lysing buffer (Tris–HCL 100 mM, NaCl 150 mM, EDTA 1 mM, Na3VO4 2 mM, NP-40 1%, protease inhibitors tablet). Lysates were precleared by centrifugation (10 min 14 000 r.p.m. at + 4°C) and incubated for 2 h at 4°C with anti-Flag (M2 clone) beads from Sigma. Beads were washed, and proteins were resuspended in NuPage LDS sample buffer (Invitrogen). Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA).

**Fixation and immunostaining**

Cells were allowed to spread on coverslips as described. Briefly, poly-lysine-covered four-chambered glass coverslips (LabTek II, Nunc/Nalgene, Rochester, NY, USA) were coated with 10 μg ml⁻¹ of antibody (anti-CD3 HIT3a or anti-CD4). The chambers were loaded with 300 μl of normal media without phenol red supplemented with 25 mM HEPES, pH 7.0 and warmed. Cells were resuspended in the same buffer, plated into the bottom of the chamber and incubated at 37°C. After 3 min, cells were fixed in 2.4% paraformaldehyde for 30 min. The cells were permeabilized with TritonX-100, incubated with blocking buffer for 30 min and then incubated with primary antibodies for 60 min, followed by washes and 60-min incubation with Alexa-conjugated secondary antibodies.

**Imaging**

Images from fixed cells were collected with a Zeiss 510 LSCM, using a × 63, 1.4 NA objective (Carl Zeiss Inc, Thornton, NY, USA). Z stacks of complete cells were taken.

**Image processing**

Zeiss AIM software (Oberkochen, Germany) was used to produce images. Adobe Photoshop and Illustrator (Adobe Systems Inc., San Jose, CA, USA) were used to prepare composite figures. Scale bars were cut from the original

---

**Figure 6**

(a) Representative flow cytometry analysis of CD45.2⁺ EGFP⁺ splenocytes from bone marrow chimeras shown in Figure 5. Upper plots show CD4 versus CD8 profiles, lower histograms show the percentage of TCRβ⁺ T cells within the CD45.2⁺ EGFP⁺ population. Plots in the left column show Themis1⁺/⁺ splenocytes infected with empty retrovector for reference. (b) Percentage of CD45.2⁺ EGFP⁺ CD4-SP and CD8-SP splenocytes in the indicated bone marrow chimeras (n=6 each). N.S., not significant (two-tailed T-test, unequal variance). All comparisons are to vector only. A full color version of this figure is available at the Immunology and Cell Biology journal online.
images and then were pasted in a more visible position on the final composite image.

ACKNOWLEDGEMENTS

We thank Jan Lee and Dalal El-Khoury for technical assistance. This work was supported by the Intramural Research Program of the Eunice Kennedy Shriver, NICHD (PEI: Project number: 1ZIAHD001803-19).

1 Lesourne R, Uehara S, Lee J, Song KD, Li L, Pinkhasov J et al. Themis, a T cell-specific protein important for late thymocyte development. Nat Immunol 2009; 10: 840–847.

2 Johnson AL, Aravind L, Shulzhenko N, Morgun A, Choi SY, Crockford TL et al. Themis is a member of a new metazoan gene family and is required for the completion of thymocyte positive selection. Nat Immunol 2009; 10: 831–839.

3 Patrick MS, Oda H, Hayakawa K, Sato Y, Eshima K, Kirikae T et al. Gasp, a Grb2-associating protein, is critical for positive selection of thymocytes. Proc Natl Acad Sci USA 2009; 106: 16345–16350.

4 Fu G, Vallee S, Rybakin V, McGuire MV, Ampudia J, Brockmeyer C et al. Themis controls thymocyte selection through regulation of T cell antigen receptor-mediated signaling. Nat Immunol 2009; 10: 848–856.

5 Kasugawa K, Yasuda T, Miura I, Kobayashi A, Fukiage H, Satoh R et al. A novel gene essential for the development of single positive thymocytes. Mol Cell Biol 2009; 29: 5128–5135.

6 Lesourne R, Zvezdova E, Song KD, El-Khoury D, Uehara S, Barr VA et al. Interchangeability of Themis1 and Themis2 in thymocyte development reveals two related proteins with conserved molecular function. J Immunol 2012; 189: 1154–1161.

7 Paster W, Brockmeyer C, Fu G, Simister PC, de Wet B, Martinez-Riano A et al. GRB2-mediated recruitment of THEMIS to LAT is essential for thymocyte development. J Immunol 2013; 190: 3749–3756.

8 Fu G, Casas J, Rigaud S, Rybakin V, Lamboléz F, Brizstek J et al. Themis sets the signal threshold for positive and negative selection in T-cell development. Nature 2013; 504: 441–445.

9 Verbeek BS, Adriaansen-Slot SS, Rijksen G, Vroom TM. Grb2 overexpression in nuclei and cytoplasm of human breast cells: a histochemical and biochemical study of normal and neoplastic mammary tissue specimens. J Pathol 1997; 183: 195–203.

10 Sorkin A, McClure M, Huang F, Carter R. Interaction of EGF receptor and grb2 in living cells visualized by fluorescence resonance energy transfer (FRET) microscopy. Curr Biol 2000; 10: 1395–1398.

11 Romero F, Ramos-Morales F, Dominguez A, Rios RM, Schweighoffer F, Tocque B et al. Grb2 and its apoptotic isoform Grb3-3 associate with heterogeneous nuclear ribonucleoprotein C, and these interactions are modulated by poly(U) RNA. J Biol Chem 1998; 273: 7776–7781.

12 Blanchet F, Cardona A, Letimier FA, Hershfield MS, Acuto O. CD28 costimulatory signal induces protein arginine methylation in T cells. J Exp Med 2005; 202: 371–377.

13 Bunnell SC, Barr VA, Fuller CL, Samelson LE. High-resolution multicolor imaging of dynamic signaling complexes in T cells stimulated by planar substrates. Science STKE 2003; 177: PL8.