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Interchangeability of Themis1 and Themis2 in Thymocyte Development Reveals Two Related Proteins with Conserved Molecular Function

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Themis1, a recently identified T cell protein, has a critical function in the generation of mature CD4+CD8− and CD4−CD8+ (CD4 and CD8 single-positive [SP]) thymocytes and T cells. Although Themis1 has been shown to bind to the adaptor proteins LAT and Grb2, previous studies have yielded conflicting results regarding whether they mediate TCR-mediated signaling defects. In this study, we demonstrate that, in the absence of Themis1, TCR-mediated signaling is selectively impaired in CD4 SP and CD8 SP thymocytes but is not affected in CD4+CD8+ double-positive thymocytes despite high expression of Themis1 in double-positive thymocytes. Like Themis1, Themis2, a related member of the Themis family, which is expressed in B cells and macrophages, contains two conserved cysteine-based domains, a proline-rich region, and a nuclear localization signal. To determine whether Themis1 and Themis2 can perform similar functions in vivo, we analyzed T cell development and TCR-mediated signaling in Themis1−/− mice reconstituted with either Themis1 or Themis2 transgenes. Notably, Themis1 and Themis2 exhibited the same potential to restore T cell development and TCR-mediated signaling in Themis1−/− mice. Both proteins were tyrosine phosphorylated and recruited within Grb2 signaling complexes to LAT following TCR engagement. These results suggest that conserved molecular features of the Themis1 and Themis2 proteins are important for their biological activity and predict that Themis1 and Themis2 may perform similar functions in T and B cells, respectively. The Journal of Immunology, 2012, 189: 1154–1161.

Several independent groups recently reported the initial characterization of a new T cell protein, E43004N04Rik, designated Themis1 (1–5). Analysis of Themis1−/− mice, generated either by gene targeting or by induced mutagenesis, identified an important role for Themis1 in T cell development: in the absence of Themis1, positive and negative selection of thymocytes is markedly impaired, and numbers of CD4+CD8− and CD4−CD8+ (CD4 or CD8 single-positive [SP]) thymocytes and T cells are strongly reduced. Despite the profound developmental defects observed in Themis1−/− mice, the molecular function of Themis1 remains unclear.

Themis1 contains two novel cysteine-based (CABIT) domains (2), a bipartite nuclear localization sequence (NLS), and a proline-rich region (PRR). In thymocytes, Themis1 is localized in both the cytoplasm and the nucleus, suggesting that it may have functions in both cellular compartments (1). Themis1 has been shown to bind to the ubiquitous cytoplasmic adaptor protein Grb2 (1, 5); however, the role of this interaction for Themis1 function has not been elucidated. Following TCR engagement, complexes formed by Grb2 and the exchange factors Vav1 and/or Sos1 (6, 7) are recruited to the scaffolding adaptor LAT (8, 9) and contribute to the activation of the ERK and JNK/P38 signaling pathways (10, 11). One group reported a requirement for Themis1 in TCR-induced ERK phosphorylation and calcium mobilization in total thymocytes (3). However, we failed to detect any signaling defects in purified CD4+CD8+ (double-positive [DP]) thymocytes from Themis1−/− mice, even though Themis1 is highly expressed in DP cells (1). Thus, no consensus has been reached regarding the role of Themis1 in TCR-mediated signal transduction.

Themis1 is the founding member of a family of related murine proteins that includes BCO13712 (ICB-1), designated Themis2, which is expressed primarily in B cells and macrophages, and 913040H23Rik, designated Themis3, which is expressed primarily in the intestine (1–3). Mice deficient in Themis2 or Themis3 have not yet been reported and, like Themis1, the function of these proteins has not been elucidated. Themis1 and Themis2 share 29% identity and 65% homology at the amino acid level. Like Themis1, Themis2 contains 2 CABIT domains (2), a putative NLS and a PRR. Although the Themis2 PRR motif (PxxPxK) differs from that of Themis1 (RxPxPxP), Themis2 was also shown to bind to Grb2 (12). In addition, Themis2 was reported to bind constitutively to Vav1 and Lyn in the Raw264.7 cell line and to regulate LPS-dependent TNF-α secretion in primary human macrophages.

Abbreviations used in this article: DP, double-positive; hCD2, human CD2; NLS, nuclear localization sequence; PRR, proline-rich region; SH3, Src homology 3; SP, single-positive.

The online version of this article contains supplemental material.
macrophages. The NLS of Themis1 and Themis2 are also not completely conserved, and the intracellular localization of Themis2 has not been evaluated.

To ascertain whether Themis1 and Themis2 perform similar functions in vivo despite their molecular differences, we expressed Themis1 or Themis2 as transgenes under the control of the T cell-specific human CD2 (hCD2) promoter/enhancer and crossed both transgenes into the Themis1−/− background. We found that Themis1 and Themis2 exhibit the same potential to restore T cell development in Themis1−/− mice. Moreover, both proteins are detected in the nucleus and the cytoplasm in thymocytes and are recruited within Grb2 signaling complexes to tyrosine phosphorylated LAT following TCR engagement. We identify a selective signaling defect in the phosphorylation of Vav1, ERK, and P38 in SP thymocytes but not DP thymocytes in the absence of Themis1 and demonstrate that this defect is corrected by expression of either Themis1 or Themis2 transgenes. These findings favor a model wherein Themis1 functions to transduce signals initiated by the TCR and suggest that Themis2 may perform a similar role in B cells in response to BCR engagement.

Materials and Methods

Mice

The hCD2-Themis1 and hCD2-Themis2 transgenes were generated by substituting murine Themis1 and Themis2 coding sequences for the cDNA sequences in the construct c-CT108 (13). Previous studies have shown that the human CD2 promoter/enhancer directs the expression of transgenes in mice to the T lineage (14). AND aβTCR transgenic mice were obtained from Taconic Farms. Animal experiments were approved by the Animal Care and Use Committee of the National Institute of Child Health and Human Development (National Institutes of Health).

Cells and plasmids

Jurkat/Tag and 293T cell lines were maintained in RPMI 1640 medium supplemented with 10% FCS, penicillin, streptomycin, and 2-ME. pXS-Fyn-myc, pSX-Lck-myc, pSX-Lck Y505F-myc, and pSX-Zap70-myc have been described previously (8). PCI-neo plasmid containing the cDNA of Themis1 was used as a template to delete the PKR region of Themis1 (RxPXXP) and the N-terminal (PKR) or the C-terminal (KRRPR) parts of the NLS of Themis1. Mutagenesis reactions were performed using the GeneTailer mutagenesis kit from Invitrogen. All mutations were verified by sequencing.

Abs and reagents

Sources for Abs and reagents used in this study include the following: anti-laminB (M-20), anti-GAPDH (FL-335), recombinant Grb2 N-terminal (1–68), or C-terminal (156–199) Src homology 3 (SH3) domains (Santa Cruz Biotechnology); anti-MyC (9E10), anti-Grb2 (81), Alexa 647-conjugated anti-pERK (pT202/pY204), pF38 (pT180/pY182), and pLAT (pY171) (BD Biosciences); anti-Vav1 (mouse ascites), anti-SOS1, and anti–phospho-tyrosine (4G10) (Millipore); and anti-pVav1 (pY160) (Invitrogen). Anti-Themis1 rabbit Abs were described previously (1). To prepare anti-Themis2 Abs, peptides corresponding to residues CK1SVHKKDRKPKNPQTNQ of mouse Themis2 were coupled to keyhole limpet hemocyanin and injected into New Zealand White rabbits (Covance). Anti-Themis2 Abs were affinity purified from rabbit serum using a covalently bound Ag column.

Subcellular fractionation

A total of 107 thymocytes or 2 × 106 Jurkat T cells were incubated in 120 μl hypotonic buffer (100 mM HEPES, 10 mM KCl, 1 mM EDTA, 2 mM Na3VO4, and protease inhibitors tablet [Roche]) for 20 min on ice. After incubation, 1.2 μl 10% Nonidet P-40 was added. Lysates were mixed vigorously and centrifuged at 3000 rpm for 5 min. Supernatants, which contained plasma membrane and cytosol, were collected. Pellets were washed with hypotonic buffer and incubated with 50 μl nuclear lysis buffer (100 mM HEPES, 400 mM NaCl, 1 mM EDTA, 2 mM Na3VO4, and protease inhibitors) for 10 min on ice. Lysates were centrifuged at 14,000 rpm for 10 min. Supernatants, which contain nuclear extract, were collected.

Intracytoplasmic staining and flow cytometry

Thymocytes (1.6 × 106/ml) were stimulated for 1 min with preformed complexes consisting of anti-CD3ε (145-2C11) plus anti-CD5 (GK1.5) (60 μg/ml) plus avidin (30 μg/ml). An excess of Cytofix/ Cytoperm buffer (BD Biosciences) was added to each tube to stop stimulations. After centrifugation, cells were resuspended in Permwash buffer (BD Biosciences), incubated 10 min at room temperature, centrifuged, and resuspended in Permwash buffer containing fluorescent Abs. Acquisition was performed on a BD Biosciences Immunocytometry Systems FACSCalibur with standard CellQuest software.

In vitro binding experiments and immunoprecipitations

Lysates from unstimulated or stimulated thymocytes were prepared as previously described (1) and incubated with recombinant Grb2 protein for 90 min at 4°C. For immunoprecipitations, anti-Themis1 or anti-Themis2 Abs were incubated for 2 h at room temperature with protein A-conjugated agarose beads. Beads were washed three times with lysing buffer and incubated with thymocyte extracts for 2 h at 4°C. Beads were washed and resuspended in NuPage sample buffer (Invitrogen). Proteins were resolved by SDS-PAGE and transferred to Immobilon-P membrane (Millipore).

Spreading assay and confocal analysis

Spreading assays were performed as described previously (15). Briefly, chambered covergrips (LabTek) were coated overnight at 4°C with the stimulatory Ab human anti-CD3ε (HTT3a or UCHT at 10 mg/ml). Cells were plated onto coated coverslips containing imaging buffer (RPMI 1640 without phenol red, 10% FCS, and 20 mM HEPES). Cells were fixed at different time points with 2.4% parafomaldehyde. Immunostaining was performed as described previously (16). Fluorescent images of fixed samples were acquired on a 510 laser-scanning confocal microscope system by using a ×63 plan apochromatic objective (Carl Zeiss).

Results

Themis1 and Themis2 transgenes can rescue T cell development in Themis1−/− mice

To determine whether Themis1 and Themis2 have redundant functions in vivo, we examined whether both proteins could independently rescue T cell development when transgenically expressed in Themis1−/− mice. Western blotting of total thymocytes from Themis1−/−; Themis2−/− mice expressing Themis1 or Themis2 transgenes (hereafter designated Themis1−/−;Themis1tg and Themis1−/−;Themis2tg, respectively) demonstrated that the Themis1 and Themis2 transgene encoded proteins were expressed in thymocytes (Fig. 1A). Surface staining for CD4 and CD8 revealed similar profiles on thymocytes and peripheral lymphoid T cells in Themis1−/−; Themis1−/−;Themis1tg and Themis1−/−;Themis2tg mice. Significantly, whereas CD4 SP and CD8 SP thymocyte and T cell numbers were strongly reduced in Themis1−/− mice, they were restored to normal levels (i.e., equivalent to those in Themis1−/−;Themis1tg and Themis1−/−;Themis2tg mice (Fig. 1B). In addition, differentiation of CD4 SP and CD8 SP thymocytes from the immature CD24hi to the mature CD24lo stage thymocytes was fully restored when either Themis1 or Themis2 transgenes were expressed in Themis1−/− mice (Supplemental Fig. 1A). We previously reported that surface expression of CD5, which parallels and is directly regulated by TCR signal intensity (17), is reduced on CD4 SP thymocytes in Themis1−/− mice. Notably, CD5 surface levels were restored to normal in Themis1−/−;Themis1tg and Themis1−/−;Themis2tg mice (Supplemental Fig. 1B; data not shown). Finally, the percentage of memory-like (CD44hiCD62Llo) T cells, which is increased in Themis1−/−;Themis1tg and Themis1−/−;Themis2tg mice (data not shown).

Although both the Themis1 and Themis2 transgenes restored T cell development in a polyclonal TCR repertoire, it was possible that they might exhibit different potentials to restore positive
We previously found that positive selection is dramatically impaired in Themis1+/− mice expressing the class II restricted ab TCR transgene (1). When either the Themis1 or the Themis2 transgene was expressed in AND TCR transgenic Themis1+/− mice, positive selection was fully restored (Fig. 2).

Collectively, these results demonstrate that Themis2 is capable of substituting for Themis1 during T cell development, indicating that Themis1 and Themis2 have redundant molecular functions in thymocytes.

Themis1 and Themis2 are localized in both the nucleus and the cytosol in thymocytes and are tyrosine phosphorylated in response to TCR engagement

We reasoned that because Themis2 is capable of restoring T cell development in Themis1+/− mice, then conserved motifs/domains between Themis1 and Themis2 would likely be functionally important. Using subcellular fractionation, we previously reported that Themis1 constitutively localizes to both the nucleus and the cytosol in thymocytes (1). To confirm that the nuclear localization of Themis1 was dependent on the bipartite NLS, we deleted either the first or the second NLS domain by mutagenesis and analyzed Themis1 cellular distribution in Jurkat T cells. Themis1 proteins lacking either domain of the bipartite NLS were undetectable in the nucleus demonstrating a requirement for this sequence for nuclear localization (Fig. 3A). Moreover, we found that the nuclear/cytoplasmic distribution of Themis1 remained unchanged in thymocytes following stimulation with PMA plus ionomycin or following stimulation with anti-TCR Abs (data not shown). The NLS of Themis1 and Themis2 are not fully conserved: PKR(X)12KRRPR for Themis1 and VPR(X)12KRRPR for Themis2. Nevertheless, like Themis1, transgenic Themis2 was also detected in the nucleus as well as the cytoplasm in unstimulated thymocytes (Fig. 3B).

Themis1 is tyrosine phosphorylated following TCR engagement (3, 18). We found that transgenic Themis2 is also rapidly tyrosine phosphorylated in thymocytes following stimulation with anti-CD3 plus anti-CD4 (Fig. 4A). In addition, Themis2 was tyrosine phosphorylated in B cells following BCR stimulation with F(ab′)2 anti-IgM Abs (Fig. 4A). Previous reports indicate that the phosphorylation of Themis1 and Themis2 is controlled by Src family kinases. Lck silencing in Jurkat T cells was shown to impair Themis1 phosphorylation (18), and Lyn was reported to bind constitutively to Themis2 in the Raw264.7 macrophages cell line (12). Cotransfection experiments in 293T cells demonstrated that Src kinases (Lck or Fyn) can phosphorylate both Themis1 and

FIGURE 1. Themis1 or Themis2 transgenic expression restores T cell development in Themis1−/− mice. (A) Analysis of Themis1 and Themis2 expression by Western blot in Themis1−/−, Themis1+/−, Themis1+/−;Themis2tg, and Themis1−/−;Themis2−/− thymocytes. (B) Analysis of Themis1+/+, Themis1−/−, Themis1−/−;Themis1tg, or Themis1−/−;Themis2tg thymocytes and lymph nodes T cells by flow cytometry. Two parameter dot plots show CD4 versus CD8 surface staining. Numbers indicate percentage of cells in the indicated quadrant. Bar graphs represent average cell numbers of the indicated thymocyte subsets calculated from six mice per group. (1) Themis1+/+, (2) Themis1−/−, (3) Themis1−/−;Themis1tg, and (4) Themis1−/−;Themis2tg. Only significant differences are noted in the graphs. ∗p < 0.05, **p < 0.01.

FIGURE 2. Themis1 or Themis2 transgene expression restores positive selection in AND TCR transgenic Themis1−/− mice. Analysis by flow cytometry of thymocytes and lymph nodes T cells in Themis1+/+, Themis1−/−, Themis1−/−;Themis1tg, and Themis1−/−;Themis2tg mice expressing MHC class II-restricted (AND) TCR. Representative two parameter plots (n = 4) show CD4 versus CD8 staining on total thymocytes or lymph node cells. Numbers indicate percentage of cells in the indicated quadrant. Single-parameter histogram plots show AND TCR staining (Va11) on total thymocytes or peripheral lymph node cells.
Moreover, Themis1 was phosphorylated following pervanadate treatment in Jurkat T cells deficient for ZAP70 (P116) but not in Jurkat T cells deficient for Lck (JCam1.6) (Fig. 4C).

Themis1 and Themis2 are recruited within Grb2 signaling complexes to tyrosine phosphorylated LAT following TCR engagement. The rapid TCR- or BCR-dependent tyrosine phosphorylation of Themis1 and Themis2 suggested that they may be recruited to immunoreceptor signaling complexes following T cell or B activation. Supporting this, we found that spreading of Jurkat T cells (transfected with Themis1-GFP plasmid) onto anti–CD3-coated coverslips, induced the redistribution of Themis1 into clusters that colocalize with the TCR, ZAP-70 kinase, and the transmembrane adaptor protein LAT (Fig. 5A). In Themis1+/+ or Themis1−/−;Themis2 tg thymocytes, LAT coprecipitated with both Themis1 and Themis2 following anti-CD3 plus anti-CD4 stimulation (Fig. 5B). LAT is tyrosine phosphorylated after TCR engagement and recruits molecular complexes formed by the adaptor protein Grb2 and the exchange factors Vav1 and Sos1 (8, 9). We found that Grb2 coprecipitated with both Themis1 and transgenic Themis2 in unstimulated or stimulated thymocytes (Fig. 5B). Grb2 association with Themis2 was also demonstrated in both unstimulated and stimulated B cells (Fig. 5B). Interestingly, Vav1 but not Sos1 coprecipitated with Themis1 and with Themis2 in unstimulated thymocytes and with Themis2 in B cells (Fig. 5B). Grb2 association with Themis2 was also demonstrated in both unstimulated and stimulated B cells (Fig. 5B). Interestingly, Vav1 but not Sos1 coprecipitated with Themis1 and with Themis2 in unstimulated thymocytes and with Themis2 in B cells (Fig. 5B). Grb2 binds constitutively to the PRR of Sos1 and Vav1 through its N-terminal and C-terminal SH3 domains, respectively. Deletion of the Themis1 PRR prevented its association with Grb2 in Jurkat T cells, indicating that this domain is essential for Themis1/Grb2 binding (Fig. 5C). A previous report showed that mutation of the Grb2 N-terminal SH3 domain, but not the C-terminal SH3 domain, prevents the binding of Themis1 to Grb2 (5). To confirm this observation, we analyzed the interaction of GST–fusion proteins containing either the N-terminal or the C-terminal SH3 domain of Grb2 with the exchange factors Vav1 and Sos1 (8, 9). We found that Grb2 coprecipitated with both Themis1 and transgenic Themis2 in unstimulated or stimulated thymocytes (Fig. 5B). Grb2 association with Themis2 was also demonstrated in both unstimulated and stimulated B cells (Fig. 5B). Interestingly, Vav1 but not Sos1 coprecipitated with Themis1 and with Themis2 in unstimulated thymocytes and with Themis2 in B cells (Fig. 5B). Grb2 binds constitutively to the PRR of Sos1 and Vav1 through its N-terminal and C-terminal SH3 domains, respectively. 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The role of Themis1 in TCR signaling has remained controversial because signaling defects in Themis1−/− thymocytes have been detected in one (3) but not other studies (1, 2, 5). In contrast, phosphorylation of LAT was unaffected by the absence of Themis1 in both DP and CD4 SP thymocytes, indicating that the signaling defect was upstream of LAT (Fig. 6). Importantly, when either the Themis1 or the Themis2 transgene was expressed in Themis1−/− thymocytes, phosphorylation of Vav1, ERK, and P38 was restored to levels comparable to wild type (Themis1+/+) CD4 SP (Fig. 6). No significant changes were observed for LAT, Vav1, ERK, and P38 phosphorylation when Themis1−/−;Themis1 tg and Themis1−/−;Themis2 tg were compared, indicating that Themis2 is capable of performing the same signaling functions as Themis1 in thymocytes (Fig. 6).

**Discussion**

To investigate the role of Themis1 in TCR-mediated signaling and to search for common functional domains within the related Themis1 and Themis2 proteins, we analyzed T cell development and TCR-mediated signaling in Themis1−/− mice reconstituted with Themis1 or Themis2 transgenes. We found that Themis1 and Themis2 exhibit the same potential to restore T cell development and TCR-mediated signaling when expressed as transgenes in thymocytes (Fig. 6). No significant changes were observed for LAT, Vav1, ERK, and P38 phosphorylation when Themis1−/−;Themis1 tg and Themis1−/−;Themis2 tg were compared, indicating that Themis2 is capable of performing the same signaling functions as Themis1 in thymocytes (Fig. 6).

**Themis1 or Themis2 can rescue the TCR-dependent signaling defect in Themis1−/− thymocytes**

Using total thymocyte extracts, Fu et al. (3) reported that Themis1 is required for phosphorylation of ERK following TCR engagement. However, we failed to detect any signaling defects in DP thymocytes isolated from Themis1−/− mice (1). To resolve this discrepancy, we performed an analysis of ERK phosphorylation following TCR stimulation plus CD4 engagement by intracellular staining and flow cytometry, which enabled us to examine specific thymocyte subpopulations. We again found that phosphorylation of ERK1/2 (pY202/pY204) was comparable in Themis1+/+ and Themis1−/− DP thymocytes after stimulation with preformed anti-CD3 plus anti-CD4 immune complexes (Fig. 6A). However, phosphorylation of ERK1/2 was significantly reduced in Themis1−/− CD4 SP thymocytes (Fig. 6A, 6B). We next examined TCR-induced phosphorylation of Vav1 (pY160), P38 (pT180/pY182), and LAT (pY171) by intracellular staining. Similar to ERK1/2, phosphorylation of Vav1, P38, and LAT was comparable in Themis1+/+ and Themis1−/− DP thymocytes (Fig. 6A). However, phosphorylation of Vav1 and P38 was also reduced in CD8 SP thymocytes (data not shown). In contrast, phosphorylation of LAT was unaffected by the absence of Themis1 in both DP and CD4 SP thymocytes, indicating that the signaling defect was upstream of LAT (Fig. 6).

**Figure 5.** Themis1 and Themis2 are recruited within Grb2/Vav1 signaling complexes to LAT following TCR engagement. (A) Formation of TCR activation clusters in Jurkat T cells expressing Themis1-GFP protein plated onto anti-CD3-coated coverslips. The colocalization of Themis1-GFP (green) with the TCR (red), ZAP70 (blue), and LAT (blue) were analyzed by confocal microscopy. (B) Analysis of LAT, Vav1, Sos1, and Grb2 coimmunoprecipitation with Themis1 and Themis2 in thymocytes and B cells. Thymocytes (Themis1+/+ or Themis1−/−;Themis2+/-) or B cells were stimulated with anti-CD3 + anti-CD4 Abs or pervanadate, respectively. Anti-Themis1 (thymocytes) or anti-Themis2 (thymocytes and B cells) immunoprecipitates were analyzed by Western blot with the indicated Abs. (C) The PRR of Themis1 mediates its association with Grb2. 293T cells were transfected with expression plasmids encoding Themis1-Flag, Grb2-hemagglutinin (HA), or Themis1-APPR (deleted of the RxPXXP motif) in the combinations shown. Anti-Themis1 immunoprecipitates were blotted with anti-HA and anti-Themis1 Abs. (D) Themis1 and Themis2 interact with the N-terminal and the C-terminal SH3 domains of Grb2. Thymocyte lysates from wild-type (Wt) or Themis2 transgenic mice were incubated with GST fusion protein expressing the N-terminal SH3 domain (1–68) or the C-terminal SH3 domain (156–199) of Grb2. Themis1 or Themis2 was immunoprecipitated and coprecipitation of GST-fusion proteins were analyzed by immunoblot with the indicated Abs. Results shown are representative of three experiments.

**Discussion**

To investigate the role of Themis1 in TCR-mediated signaling and to search for common functional domains within the related Themis1 and Themis2 proteins, we analyzed T cell development and TCR-mediated signaling in Themis1−/− mice reconstituted with Themis1 or Themis2 transgenes. We found that Themis1 and Themis2 exhibit the same potential to restore T cell development and TCR-mediated signaling when expressed as transgenes in Themis1−/− mice, indicating that conserved domains in these proteins are likely important for their biological activity.
The absence of any discernable signaling defects in DP thymocytes, which we favor, is that Themis1 does not participate in the initiation of TCR-mediated signaling in DP thymocytes but instead functions to sustain signals initiated during positive selection as thymocytes transition from the DP to the SP stage. A widely accepted model of CD4/CD8 lineage choice contends that this event is dictated by the intensity or duration of TCR signals, with stronger or more persistent signaling initiated by MHC class-II restricted TCRs plus CD4 promoting CD4 lineage commitment and attenuated or interrupted signaling by MHC class I-restricted TCRs in the absence of CD8 costimulation (as a consequence of stage specific CD8 downregulation) promoting CD8 lineage commitment (20). We have shown that MHC class II-restricted thymocytes are redirected to the CD8 lineage in Themis1−/− mice consistent with an impairment of signaling at the immature SP stage (1). We also observed that upregulation of CD5, CD69, and Gata-3 are not affected in positively selected DP thymocytes but are reduced in transitional CD4+CD8α thymocytes in Themis1−/− mice (1). Our current results are therefore consistent with a mechanism whereby Themis1 functions primarily to sustain signaling initiated by the TCR in transitional CD4+CD8α thymocytes, either directly or by affecting the expression or activity of other factors involved in the TCR signaling response.

Themis1 and Themis2 bind constitutively to Grb2 and to the GTP/GDP exchange factor Vav1. The phosphorylation of Vav1 and its activity (data not shown) are downregulated in Themis1−/− CD4 and CD8 SP thymocytes and are restored by re-expression of Themis1 or by expression of Themis2. Interestingly, mice conditionally deficient for Grb2 in thymocytes and “knockin” mice expressing catalytically inactive Vav1 display a phenotype strikingly similar to that of Themis1−/− mice (i.e., decreased numbers of CD4 or CD8 SP thymocytes and T cells) (21, 22). This suggests that a signaling complex formed by Themis1-Grb2-Vav1 may play a critical role during positive selection of thymocytes. Vav1 catalytic activity is required for the production of Rac1-GTP in thymocytes. Rac1-GTP in turn is important for the activation of the MAPKs JNK and P38, which have been implicated in negative selection (23–25). P38 phosphorylation was decreased in Themis1−/− CD4 and CD8 SP thymocytes, suggesting that reduced Vav1-mediated activation of P38 may explain the defect in negative selection in Themis1−/− mice (1, 3). We also found that ERK phosphorylation was reduced in Themis1−/− CD4 SP thymocytes, and sustained activation of ERK has been shown to be important for positive selection in thymocytes (26). The catalytic domain of Vav1 is not required for ERK activity, but interestingly, ERK activation is dramatically impaired in Vav1-deficient thymocytes (11). This raises the possibility that Themis1 could regulate Vav1 adaptor functions, for instance, by controlling the recruitment of Vav1 into TCR-dependent signaling complexes and thereby affect ERK activation. Although our data indicate that Themis1 and Themis2 may regulate Vav1 activity in submembranous signaling complexes, the possibility remains that they also regulate Vav1 in the nuclear compartment. Methylated forms of Vav1 have been shown to be selectively translocated to the nucleus after TCR plus CD28 engagement and to participate in IL-2 synthesis in T cells (27). Although the bipartite NLS sequence of Themis proteins is not completely conserved in Themis1 and Themis2, we found that both proteins are partly localized in the nucleus in resting cells. It is therefore possible that Themis proteins play a dual role affecting both submembranous and intranuclear signaling events in lymphocytes, although this remains to be tested experimentally.

Finally, our current data provide potential insight into the role played by Themis2 in B cells. Conditional knockout mice deficient for Grb2 in B cells and Vav1−/−/Vav2−/− mice show...
a dramatic reduction of recirculating mature B cells in the bone marrow and a strong reduction of mature B cells in the spleen and the blood (28, 29). It is possible that mice deficient for Themis2 will have a similar phenotype. However, a potential caveat to this reasoning is that analogous T and B cell signaling proteins have often been shown to have a dissymmetrical impact on T cell and B cell development, respectively. Like Themis1 and Themis2, the related adapter proteins LAT and LAB/NTAL, or SLP76 and BLNK/SLP65, are selectively expressed in T cells (8, 30) and B cells (31, 32), respectively. Although these molecules share conserved motifs/domains and are localized in similar signaling complexes, they exhibit functional/physiological differences. LAT-deficient mice display a profound block in T cell development (33), whereas B cell development appears normal in LAB/NTAL−/− mice (34, 35), even though the transgenic expression of LAT/NTAL in T cells completely restores T cell development in LAT−/− mice (36). Similarly, T cell development is completely blocked in SLP76-deficient mice (37, 38), whereas B cell development is only partially impaired in the absence of SLP65 (the homolog of SLP76 in B cells). In the latter case, it has been shown that the combined deletion of LAT and SLP65 genes severely impairs B cell development. The same study showed that LAT and SLP76 are expressed at early stages of B cell development and act cooperatively with SLP65 to promote B cell maturation (39). Themis1 expression has not been extensively examined in B cells. If expressed in immature B cell progenitors, it is possible that Themis1 could function redundantly with Themis2 to regulate early stages of B cell development.

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Disclosures

References

Figure S1: Themis1 or Themis2 transgenic expression restores CD4 SP or CD8 SP differentiation in Themis1⁻/⁻ mice. A, Analysis of Themis1⁺/⁺, Themis1⁻/⁻, Themis1⁻/⁻; Themis1tg, Themis1⁻/⁻; Themis2tg CD4 SP and CD8 SP thymocytes by flow cytometry. Two parameter dot plots show CD24 versus CD3 surface staining. Numbers indicate percentage of cells in the indicated quadrant. B, Analysis of CD5 surface staining on CD4 SP thymocytes from Themis1⁺/⁺, Themis1⁻/⁻, Themis1⁻/⁻; Themis2tg mice.