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THEMIS, a new T cell specific protein important for late thymocyte development

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Abstract

During positive selection, thymocytes transition through a stage during which T cell receptor (TCR) signaling controls CD4 versus CD8 lineage choice and subsequent maturation. Here, we describe a new T cell specific protein, THEMIS, that performs a distinct function during this stage. In *Themis*^{-/-} mice, thymocyte selection was impaired and the number of transitional CD4⁺CD8^{int} thymocytes as well as CD4 and CD8 single positive thymocytes was decreased. Remarkably, although no overt TCR-proximal signaling deficiencies were detected, *Themis*^{-/-}CD4⁺CD8^{int} thymocytes exhibited developmental defects consistent with attenuated signaling that were reversible by increased TCR stimulation. These results identify THEMIS as a critical component of the T cell developmental program and suggest that THEMIS functions to sustain and/or integrate signals required for proper lineage commitment and maturation.

Introduction

In addition to their role in thymocyte selection, signals transduced by the T cell antigen receptor (TCR) promote the transition of CD4⁺CD8⁺ (double positive, DP) thymocytes to the CD4⁻CD8⁺ (CD8SP) and CD4⁺CD8⁻ (CD4SP) stages and regulate CD4 versus CD8 lineage commitment¹. Although the importance of TCR signaling in each of these steps has been established, the precise mechanisms controlling DP thymocyte differentiation and CD4 versus CD8 lineage choice have yet to be fully defined. A current model proposes that interactions between the TCR (plus CD8 or CD4) and either MHC class I or MHC class II molecules generate developmentally equivalent signals that drive maturation of DP thymocytes to the CD4⁺CD8^{intermediate} (CD4⁺CD8^{int}) stage without directing CD4 versus CD8 lineage commitment². According to this model, lineage choice is subsequently dictated by signal intensity or duration, with stronger or more persistent signaling by MHC class II-restricted TCRs plus CD4 promoting CD4 lineage commitment and attenuated or interrupted

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signaling by MHC class I restricted TCRs in the absence of CD8 costimulation (as a consequence of CD8 down-regulation) promoting CD8 lineage commitment.

A central tenet of this model is that the maturation of DP thymocytes into SP thymocytes requires the sequential induction and activation of two classes of effectors, those that function primarily to promote positive selection and/or developmental progression, and those that regulate CD4 versus CD8 lineage determination. Indeed, experimental data have largely supported this paradigm. The transcription factors Gata-3, Tox, Egr-1 and c-Myb are induced and/or regulated by TCR signaling in DP thymocytes and are important for positive selection and transition of DP thymocytes to the CD4⁺CD8^{int} stage but do not directly regulate CD4 versus CD8 lineage commitment^{1, 3, 4}. On the other hand, Th-POK and Runx3 are first expressed at the CD4⁺CD8^{int} stage and are not required for the DP→CD4⁺CD8^{int} transition, but instead function as essential mediators of CD4 and CD8 lineage choice, respectively^{1, 3, 5}. As the distinguishing factor in CD4 versus CD8 lineage choice (signal intensity and/or duration) is by definition quantitative, it is also presumed that quantitative differences in signal output translate into the qualitative changes in gene expression required to direct lineage choice. Gata-3 appears to perform such a role as its expression is differentially regulated by TCR signaling in MHC class I and MHC class II-restricted CD4⁺CD8^{int} thymocytes⁶, and Gata-3 is required for expression of Th-POK⁷. However, in the absence of Gata-3, Th-POK is incapable of directing the maturation of CD4⁺CD8^{int} thymocytes into CD4SP thymocytes, indicating that additional, as yet undefined factors are required for CD4 lineage differentiation⁷.

In this report, we present the identification and initial characterization of a novel T cell specific protein, THEMIS. THEMIS is highly conserved in all vertebrate species and is present in the nucleus, yet lacks an identifiable catalytic domain. *Themis*^{-/-} mice exhibited a late block in thymocyte selection characterized by reduced numbers of CD4⁺CD8^{int}, CD4SP and CD8SP thymocytes. Although no proximal TCR signaling defect was detected in *Themis*^{-/-} thymocytes, CD4 lineage commitment and maturation was compromised in a manner consistent with signal attenuation suggesting that THEMIS is involved at a distal point in the TCR signaling cascade. These results indicate that THEMIS functions to integrate or sustain TCR signaling in CD4⁺CD8^{int} thymocytes and demonstrate that this capability is critical for appropriate lineage commitment as well as CD4SP and CD8SP thymocyte development.

Results

Identification and cloning of E430004N04Rik (*Themis*)

To search for new genes that may be important for T cell development, we performed a cDNA subtraction using mRNA from multipotent progenitor and T cell lineage committed fetal liver CD4⁺CD8⁻ (double negative, DN) thymocyte subsets. One cDNA clone obtained from the subtraction screen (E430004N04Rik) encoded a novel protein and was chosen for further analysis. In a consensus reached among the three groups publishing in this issue, E430004N04Rik was named *Themis* (Thymus expressed molecule involvement in selection).

The mouse *Themis* cDNA clone contains an open reading frame encoding a 636 amino acid protein (Fig. 1a and Supplementary Fig. 1). The corresponding human cDNA (C6orf190), shares 80% overall identity to the murine protein (Fig. 1a and Supplementary Fig. 1). Alignment of orthologous vertebrate sequences identified two highly conserved motifs; a bipartite nuclear localization signal (NLS), (KR-X₁₂-KRRPR), and a carboxy terminal proline-rich site (PPRPPKxP) that matches a Class II SH3 recognition motif (PxxPx+)⁸ (Supplementary Fig. 1). Several other conserved regions of unknown function were detected, but no potential catalytic domains were identified. A BLAST search identified two

related mouse proteins: BC013712 (ICB-1), which is selectively expressed in B cells, and 9130404H23Rik, which is selectively expressed in the intestine (Supplementary Fig. 2 and data not shown).

Themis mRNA was present in the thymus and in secondary lymphoid tissues (lymph nodes and spleen), but not in bone marrow (BM) or non-lymphoid tissues (Fig. 1b). THEMIS was detected in all thymocyte subsets and in all mature T cells, including both CD4SP and CD8SP $\alpha\beta$ T cells and $\gamma\delta$ T cells, but not in B cells or NK cells (Fig. 1c). In addition, THEMIS protein quantities were equivalent in wild type and *Tcra*^{-/-} thymocytes indicating that TCR signaling is not required for THEMIS expression (Fig. 1c). Intracellular staining demonstrated that THEMIS expression is highest in DP thymocytes and is down-regulated in SP thymocytes (Fig. 1d and data not shown).

THEMIS was detected in both the nucleus and the cytoplasm in thymocytes (Supplementary Fig. 3). Although THEMIS was tyrosine phosphorylated after TCR cross-linking (data not shown), protein expression and nuclear versus cytoplasmic partitioning were unaffected following TCR stimulation (Supplementary Fig. 3). The NLS was required for nuclear importation and retention, since THEMIS variants in which either the N- or C-terminal conserved NLS sequences were mutated were not detected in the nucleus (data not shown).

Using an SH3 domain array screen we identified the cytoplasmic adaptor Grb2 as a potential THEMIS binding partner. Co-immunoprecipitation experiments demonstrated that THEMIS binds Grb2 but not the related family members Gads or Grap (Supplementary Fig. 3 and data not shown). Although association of THEMIS with Grb2 did not require TCR stimulation, we consistently detected increased THEMIS-Grb2 association in thymocytes subjected to TCR cross-linking; these findings suggest that the THEMIS-Grb2 interaction may be mediated by both the SH2 and SH3 domain(s) of Grb2 (data not shown).

Block in late T cell development in *Themis*^{-/-} mice

To evaluate the role of THEMIS in T cell development, exon 1 of the *Themis* gene was deleted by homologous recombination in embryonic stem cells (Supplementary Fig. 4). *Themis* mRNA and protein were undetectable in thymocytes from *Themis*^{-/-} mice, confirming that the induced mutation resulted in a null allele (Supplementary Fig. 4 and data not shown). *Themis*^{-/-} mice were viable and fertile and exhibited no overt signs of immune deficiency. Thymus size and cellularity were similar in *Themis*^{+/+}, *Themis*^{+/-} and *Themis*^{-/-} mice (Supplementary Fig. 5). Immature DN thymocyte subsets and total DN as well as DP thymocyte percentages and numbers were similar in *Themis*^{-/-} and control littermates indicating that THEMIS is not required for β -selection or for maturation from the DN to the DP stage (Fig. 2a and Supplementary Fig. 5). Moreover, surface expression of the TCR and of the maturation and activation markers CD2, CD24, CD5 and CD69 was comparable on *Themis*^{+/+} and *Themis*^{-/-} DP thymocytes (Supplementary Fig. 5 and data not shown). However, CD4SP and CD8SP thymocyte numbers were reduced in *Themis*^{-/-} mice in comparison to *Themis*^{+/+} and *Themis*^{+/-} littermates (Fig. 2a and data not shown). In addition, CD4SP thymocyte differentiation was markedly impaired in *Themis*^{-/-} mice as the ratio of mature (CD24^{lo}) to immature (CD24^{hi}) TCR^{hi}CD4⁺ thymocytes was strongly reduced (Fig. 2c). Using the same criteria, CD8SP differentiation appeared less severely affected (Fig. 2c).

Peripheral CD4⁺ and CD8⁺ T cell numbers were reduced in *Themis*^{-/-} mice, and a higher percentage of T cells displayed a memory phenotype (CD44^{hi}CD62L^{lo}), possibly due to lymphopenia-driven population expansion (Fig. 2b and Supplementary Fig. 5). *Themis*^{+/+} and *Themis*^{-/-} peripheral T cells showed similar proliferative responses to TCR cross-linking, indicating that low numbers of mature, functional T cells can be generated in the absence of THEMIS (Supplementary Fig. 5). Evaluation of other T lymphoid populations revealed that

THEMIS is not essential for the development of $\gamma\delta$ T cells, NKT cells or regulatory T cells, although CD4⁺ regulatory T cell numbers were significantly reduced in *Themis*^{-/-} mice (Supplementary Fig. 5).

To determine if the developmental defect in *Themis*^{-/-} mice was T cell autonomous, chimeric mice were generated by injecting a 4:1 (*Themis*^{-/-}(CD45.2⁺):B6 (CD45.1⁺)) mixture of BM cells into irradiated B6:CD45.1⁺ congenic mice. Thymocytes derived from *Themis*^{-/-} BM exhibited a phenotype identical to that of thymocytes in germline *Themis*^{-/-} mice, demonstrating that the defect was T cell intrinsic (Supplementary Fig. 6).

THEMIS is required for positive selection

The reduction in SP but not DP thymocytes in *Themis*^{-/-} mice suggested a block in positive selection. To evaluate positive selection, MHC class-I restricted (H-Y) and MHC class-II restricted (AND) $\alpha\beta$ -TCR transgenes were introduced into the *Themis*^{-/-} background. Under conditions that promote positive selection, DP thymocytes in H-Y *Themis*^{-/-} and AND *Themis*^{-/-} mice exhibited normal induction of CD69 and CD5 expression (Fig. 3a and data not shown). However, H-Y *Themis*^{-/-} and AND *Themis*^{-/-} mice contained significantly fewer CD8SP and CD4SP thymocytes, respectively, than their corresponding TCR transgenic *Themis*^{+/+} littermates (Fig. 3a). Thus, *Themis*^{-/-} DP thymocytes were capable of initiating but not completing positive selection. DP thymocyte numbers were increased in all *Themis*^{-/-} TCR transgenic mice relative to *Themis*^{+/+} TCR transgenic controls suggesting that the reduction in SP thymocytes was caused by a block in positive selection rather than conversion to negative selection (Fig. 3a).

We also evaluated thymocyte selection by comparing gene expression in CD69⁺ thymocytes from *Themis*^{+/+} and *Themis*^{-/-} mice. Microarray profiling revealed significant differences in expression of 132 genes (Supplementary Fig. 7). Among these were seventeen genes that had been shown previously to be transcriptionally regulated (either induced or repressed) during positive selection^{9, 10}. The change in expression of these genes in *Themis*^{-/-} versus *Themis*^{+/+} thymocytes was also strongly suggestive of a block in positive selection (Supplementary Fig. 8).

To evaluate negative selection, we analyzed thymocytes from male H-Y TCR transgenic mice, which undergo early deletion at the DN or DP stage in the presence of a male-specific self antigen¹¹. Thymocyte numbers were strongly reduced in H-Y *Themis*^{-/-} males compared to corresponding H-Y female littermates demonstrating that H-Y TCR⁺ thymocytes are negatively selected in the absence of THEMIS (Fig. 3b). However, the percentage of DP thymocytes was consistently higher in H-Y *Themis*^{-/-} males compared to H-Y *Themis*^{+/+} males suggesting that negative selection occurs less efficiently in the absence of THEMIS (Fig. 3b). In addition, Staphylococcus Enterotoxin B (SEB) superantigen induced deletion of V β 8⁺ CD4SP thymocytes but not V β 8⁺ CD8SP thymocytes was impaired in the absence of THEMIS (Supplementary Fig. 9), suggesting a requirement for THEMIS in late negative selection of CD4SP thymocytes.

Fewer CD4⁺CD8^{int} thymocytes in *Themis*^{-/-} mice

To identify the point where thymocyte development is first affected in *Themis*^{-/-} mice, total thymocytes were stained with CD3 and CD69 (Fig. 4a). The numbers of CD3^{lo}CD69⁻ cells and CD3^{int}CD69⁺ cells, which represent pre-selection DP thymocytes and DP thymocytes at the initial stage of positive selection, respectively, were normal in *Themis*^{-/-} mice (Fig. 4a). However, the numbers of CD3^{hi}CD69⁺ and CD3^{hi}CD69⁻ thymocytes, which include primarily immature and mature SP thymocytes, respectively, were reduced (Fig. 4a).

Following initiation of positive selection, DP thymocytes pass through a CD4⁺CD8^{int} transitional stage before committing to either the CD4 or the CD8 lineage^{2, 12-14}. The number of (CD3^{hi}) CD4⁺CD8^{int} cells, but not their precursors, CD69⁺DP thymocytes, was reduced in *Themis*^{-/-} mice (data not shown). The reduction in CD4⁺CD8^{int} thymocytes was confirmed in MHC II-deficient (I-A^{β-/-}) *Themis*^{-/-} mice (Fig. 4b), where, due to the absence of mature CD4SP thymocytes, the CD4⁺CD8^{int} thymocyte population is highly enriched for transitional cells².

To determine if a similar defect could be observed *in vitro*, we performed a two stage differentiation assay¹⁵. Although antibody stimulation of purified *Themis*^{-/-} DP thymocytes resulted in normal up-regulation of CD69, down-regulation of CD8 was impaired and fewer CD4⁺CD8^{int} cells were generated in the recovery culture (Fig. 4c and data not shown). These findings point to a defect in the generation or survival of CD4⁺CD8^{int} thymocytes. Expression of the pro-survival proteins Bcl-2 and Bcl-xL was unaffected in *Themis*^{-/-} DP and CD4⁺CD8^{int} thymocytes and no survival defect was found during *in vitro* culture (Supplementary Fig. 10). In addition, staining with Ki-67 and DAPI demonstrated that there were no obvious cell cycle abnormalities in *Themis*^{-/-} thymocyte subpopulations (Supplementary Fig. 10). Interestingly, CD8SP thymocytes and T cells in *Themis*^{-/-} mice expressed higher amounts of surface CD8 than comparable populations from *Themis*^{+/+} mice (Supplementary Fig. 11 and data not shown). However, as deletion of CD8 did not rescue CD4SP development in *Themis*^{-/-} mice, the reduction in CD4SP thymocytes could not be attributed to a defect in CD8 down-regulation (Supplementary Fig. 11).

Signaling defect in *Themis*^{-/-} CD4⁺CD8^{int} thymocytes

The block in selection in *Themis*^{-/-} mice pointed to a defect in TCR signaling; however, *Themis*^{-/-} DP thymocytes expressed normal surface quantities of the activation markers CD5 and CD69 (Fig. 3a and Supplementary Fig. 5) and no impairment in the activation and signaling responses of *Themis*^{-/-} DP thymocytes was observed, even when stimulation conditions were varied (Supplementary Fig. 12 and data not shown). In contrast to DP thymocytes, CD4⁺CD8^{int} thymocytes from *Themis*^{-/-} mice expressed lower amounts of CD5 and CD69 than corresponding cells from *Themis*^{+/+} mice (Fig. 5a). In addition, surface expression of the IL-7 receptor, which is also directly regulated by TCR signal intensity^{16, 17}, was decreased on *Themis*^{-/-} CD4⁺CD8^{int} thymocytes (Fig. 5a). TCR (CD3ε) surface expression was equivalent on CD4⁺CD8^{int} thymocytes from *Themis*^{+/+} and *Themis*^{-/-} mice; thus, the reduced expression of CD5 and CD69 could not be attributed to lower surface TCR quantities (Fig. 5a).

Cross-linking of TCRs on CD4⁺CD8^{int} thymocytes from *Themis*^{-/-} mice elicited normal calcium flux, and normal phosphorylation of the kinases ZAP-70 and Erk (Supplementary Fig. 12). Thus, as in DP thymocytes, proximal TCR signaling responses did not appear to be affected in *Themis*^{-/-} CD4⁺CD8^{int} thymocytes. Since TCR signaling appeared qualitatively normal in *Themis*^{-/-} thymocytes, we considered the possibility that signal intensity or duration may be affected. CD4 thymocyte maturation is critically dependent upon strong and/or persistent signaling^{1, 18, 19}, and attenuation or cessation of TCR signaling at the CD4⁺CD8^{int} stage results in the 're-direction' of MHC class II restricted thymocytes into the CD8 lineage^{2, 20}. Similar to MHC^{+/+}*Themis*^{-/-} mice, surface expression of CD5 and CD69 was reduced on CD4⁺CD8^{int} thymocytes in MHC I^{-/-} (β2m^{-/-}) *Themis*^{-/-} mice (Fig. 5b). Notably, CD3^{hi} CD8SP thymocytes were present in MHC I^{-/-}*Themis*^{-/-} mice but not in control MHC I^{-/-}*Themis*^{+/+} mice (Fig. 5b). The majority of these CD8SP thymocytes were TCR^{hi}CD69⁺ indicating that they received positively selecting signals by interaction with self-MHC class-II complexes but were subsequently 'redirected' to the CD8 lineage (Fig. 5b).

A critical endpoint of TCR signaling during positive selection is the regulated expression of nuclear factors that control thymocyte differentiation and CD4 versus CD8 lineage commitment. The transcription factors Tox and Egr-1, which are induced in DP thymocytes by TCR engagement during thymocyte selection^{21, 22}, were expressed in similar quantities in CD69⁺ thymocytes in *Themis*^{+/+} and *Themis*^{-/-} mice (Fig. 6a). However, Gata-3 and Th-POK, transcription factors selectively required for CD4SP thymocyte development^{6, 23, 24}, were present in markedly reduced amounts in *Themis*^{-/-} CD69⁺ thymocytes (Fig. 6a). In wild-type thymocytes, Gata-3 is induced in CD69⁺ DP cells and is then further up-regulated in a subset of CD4⁺CD8^{int} thymocytes that are undergoing CD4 lineage differentiation (Fig. 6b)⁶. Interestingly, Gata-3 was normally up-regulated in CD69⁺ DP thymocytes from *Themis*^{-/-} mice (Fig. 6b). However, *Themis*^{-/-} CD69⁺CD4SP thymocytes did not contain a Gata-3^{hi} population (Fig. 6b). Th-POK is first expressed in CD4⁺CD8^{int} thymocytes and is specific to cells that are undergoing CD4 lineage differentiation²⁴. Like Gata-3, Th-POK was induced in *Themis*^{-/-} CD69⁺CD4SP thymocytes; however, its expression was reduced relative to that in *Themis*^{+/+} CD69⁺CD4SP thymocytes (Fig. 6c).

CD4SP differentiation rescued by TCR cross-linking

Since Th-POK and Gata-3 are both induced by TCR signaling^{6, 25}, their reduced expression in *THEMIS*^{-/-} CD4⁺CD8^{int} thymocytes could be a consequence of attenuated TCR signaling. Alternatively, THEMIS could be specifically required for the expression of Gata-3 and/or Th-POK at the CD4⁺CD8^{int} stage. To determine if normal expression of Gata-3 and Th-POK could be restored in *THEMIS*^{-/-} thymocytes by enhanced TCR signaling, *Themis*^{-/-} MHC class II^{-/-} mice were injected with anti-TCR β . CD4⁺CD8^{int} thymocytes in MHC class II^{-/-} mice expressed low amounts of Gata-3 and Th-POK and CD4SP thymocytes are not generated in these mice (Fig. 7a,b). However, *in vivo* stimulation with anti-TCR β induces Th-POK expression and CD4SP differentiation^{25, 26}. Four days after injection, antibody treated *Themis*^{+/+} and *Themis*^{-/-} mice contained similar numbers of CD4SP thymocytes, which expressed high and comparable amounts of CD5 and CD69 (Fig. 7a,b and data not shown). Notably, Gata-3 and Th-POK were strongly induced and their expression was similar in *Themis*^{+/+} and *Themis*^{-/-} thymocytes (Fig. 7c). These findings demonstrate that enhanced TCR signaling can reverse the transcriptional defects caused by THEMIS deficiency.

To determine if the block in CD4SP thymocyte development in *Themis*^{-/-} mice could be reversed by restoring high Gata-3 expression in CD4⁺CD8^{int} thymocytes, we introduced a T cell-lineage specific Gata-3 transgene into the *Themis*^{-/-} background. Although Gata-3 expression was increased to the high-normal range in *THEMIS*^{-/-} Gata-3 transgenic CD4⁺CD8^{int} thymocytes (Supplementary Fig. 13), the block in CD4SP thymocyte development was neither reversed nor alleviated (Fig. 8a). In addition, despite the restoration of high Gata-3 expression, Th-POK expression was not induced in *Themis*^{-/-} Gata-3 transgenic CD69⁺ thymocytes (Supplementary Fig. 13). Forced expression of Th-POK also failed to alleviate the CD4SP developmental block in *Themis*^{-/-} mice, even though overexpression of Th-POK blocked the development of CD8SP thymocytes (Fig. 8b). Thus, *Themis*^{-/-} thymocytes responded to the CD8 lineage inhibiting effects of Th-POK, but Th-POK was incapable of rescuing the block in CD4 lineage development. Taken together, these findings indicate that the reduced expression of Gata-3 and Th-POK is due to reduced TCR signaling in *Themis*^{-/-} CD4⁺CD8^{int} thymocytes and suggest that the defect in CD4SP maturation is due to the defect in TCR signaling rather than solely to a specific defect in the expression of Gata-3 or Th-POK.

Impaired survival of *Themis*^{-/-} CD8SP thymocytes

To determine if the block in CD8SP maturation in *Themis*^{-/-} mice is associated with a transcriptional defect, we examined expression of Runx3 and c-Myb, transcription factors whose up-regulation or down-regulation, respectively, is important for CD8SP development^{27, 28}. Notably, THEMIS was not required for appropriate regulation of Runx3 and c-Myb in MHC class I restricted CD69⁺ thymocytes (Supplementary Fig. 14). These findings are consistent with previous data indicating that strong and/or sustained TCR signaling is not critical for the regulated expression of transcription factors controlling CD8 development²⁰.

Although CD8SP thymocyte differentiation does not require strong and/or sustained TCR signaling, both CD4⁺CD8^{int} and immature CD8SP thymocytes are still dependent upon TCR signals for survival via the direct induction of Bcl-2 and the induction of IL-7 receptor expression^{2, 29, 30}. To determine if CD8SP development could be rescued in *Themis*^{-/-} mice by promoting cell survival, we introduced a Bcl-2 transgene into *Themis*^{-/-} mice. Notably, the Bcl-2 transgene increased the number of CD4⁺CD8^{int} and CD8SP thymocytes in *Themis*^{-/-} mice to an extent comparable to that in control *Themis*^{+/-} mice (Fig. 9a, b). As expected, the Bcl-2 transgene was not sufficient to rescue CD4SP development in *Themis*^{-/-} mice (Fig. 9b). IL-7 receptor signaling was not impaired in the absence of THEMIS since addition of IL-7 increased the survival of *Themis*^{-/-} immature CD8SP thymocytes during *in vitro* culture (data not shown). Collectively, these and the previous results indicate that the reduction in CD4⁺CD8^{int} and CD8SP thymocytes in *Themis*^{-/-} mice is caused by a survival defect secondary to attenuated TCR signaling.

Discussion

In this study, we relate the identification and initial characterization of THEMIS, a novel T cell specific protein identified by subtractive cloning. Structurally, THEMIS most closely resembles an adapter or linker due to the absence of a defined catalytic domain. THEMIS contains a proline rich motif that mediates its constitutive association with the ubiquitous adapter protein Grb2. Grb2 has been shown to regulate p38 and Jnk MAP kinase signaling in thymocytes, and negative selection is compromised in *Grb2*^{+/-} mice³¹; thus THEMIS might regulate Grb2 function in thymocytes. However, we found no defect in Jnk or Erk activation in *Themis*^{-/-} thymocytes, and the developmental block was not exacerbated in *Themis*^{-/-} *Grb2*^{+/-} mice (data not shown). Thus, the physiological importance of the THEMIS-Grb2 association remains to be elucidated.

Themis^{-/-} mice exhibited two striking defects in late thymocyte development. First, the number of transitional CD4⁺CD8^{int} thymocytes was significantly reduced. As transitional CD4⁺CD8^{int} thymocytes contain both CD4 and CD8 lineage precursors^{2, 12-14}, their reduction in *Themis*^{-/-} mice provides an explanation for the reduction in both CD4SP and CD8SP thymocytes as well as peripheral T cells. Second, CD4⁺CD8^{int} thymocytes in *Themis*^{-/-} mice were impaired in their ability to differentiate into mature CD4SP or CD8SP thymocytes. Although forced expression of Bcl-2 restored normal numbers of CD4⁺CD8^{int} and CD8SP thymocytes in *THEMIS*^{-/-} mice, CD4SP development was not rescued by Bcl-2, revealing defects in *Themis*^{-/-} CD4⁺CD8^{int} thymocytes beyond cell survival.

Despite intensive investigation, we were unable to attribute the block in development to the reduced activation of any specific signaling effector or transcription factor. Several genes known to be regulated during positive selection displayed altered expression patterns in *Themis*^{-/-} CD69⁺ thymocytes and the change in their expression (up or down) relative to *Themis*^{+/-} thymocytes is most consistent with developmental immaturity. The apparent discrepancy between the phenotype of *Themis*^{-/-} thymocytes and the lack of a proximal TCR

signaling defect could indicate that THEMIS is required for transduction of non-TCR mediated signals that are important for CD4SP thymocyte maturation. However, no defects in chemotaxis, integrin-dependent adhesion, or IL-7 receptor, Wnt and Notch signaling were discernable in *Themis*^{-/-} thymocytes (data not shown). The fact that augmented TCR signaling was capable of restoring normal expression of Gata-3 and Th-POK in *Themis*^{-/-} thymocytes indicates that if there is a defect in a parallel signaling pathway it can be bypassed by enhancing TCR signaling. An alternative explanation which we favor is that THEMIS functions downstream of the TCR to integrate or sustain TCR signals. THEMIS is present in the nucleus, indicating that it could regulate the activity or expression of nuclear factors involved in SP thymocyte development. Sustained signaling has been shown to be essential for CD4SP thymocyte development,^{1, 18, 19} and CD4SP thymocyte maturation was more severely impacted in *Themis*^{-/-} mice than CD8SP development. Although profound, the block in CD4SP thymocyte development was not absolute, suggesting that compensatory factors such as an increase in the affinity of the positively selected repertoire may facilitate the development of low numbers of CD4SP in *Themis*^{-/-} mice. Consistent with attenuated signaling, MHC class II restricted CD4⁺CD8^{int} thymocytes in *THEMIS*^{-/-} mice express reduced surface amounts of CD5 and CD69 and exhibited defects in induction of Gata-3 and Th-POK, both of which are regulated by TCR signaling^{6, 25}. In addition, *Themis*^{-/-} MHC class II restricted CD4⁺CD8^{int} thymocytes were 're-directed' into the CD8 lineage, an event that has been directly linked to TCR signal attenuation^{1, 2, 20}. The rescue of CD8 development in *Themis*^{-/-} mice by overexpression of Bcl-2 suggests that the main impact of attenuated signaling on *Themis*^{-/-} MHC class I restricted CD4⁺CD8^{int} thymocytes is reduced cell survival, presumably due to suboptimal induction of Bcl-2. The fact that Bcl-2 expression was normal in *Themis*^{-/-} CD4⁺CD8^{int} thymocytes does not exclude this possibility since cells that fail to normally induce Bcl-2 would presumably undergo apoptosis and be rapidly cleared in the thymus.

The 're-direction' of MHC class II restricted *Themis*^{-/-} thymocytes to the CD8 lineage demonstrates that the block in thymocyte development occurs prior to CD4 lineage commitment. Supporting this contention is the fact that although forced expression of Th-POK did not reverse the block in CD4 development, it effectively blocked CD8 lineage commitment and development in *Themis*^{-/-} mice. A similar effect was observed when a Th-POK transgene was introduced into mice in which Gata-3 was conditionally inactivated at the DP stage (ΔDP), demonstrating that Th-POK can antagonize CD8SP development independent of Gata-3⁷.

As is the case for *Themis*^{-/-} mice, thymocyte development is also blocked prior to CD4 versus CD8 lineage commitment in both *Tox*^{-/-} and Gata-3(ΔDP) mice^{7, 32}. Lineage 're-direction' is observed in all three knockouts, albeit to different extents, and in all three knockouts CD4 development is impacted to greater extent than CD8 development. However, whereas transitional CD4⁺CD8^{int} thymocytes were reduced in *Themis*^{-/-} and Gata-3(ΔDP) mice, they are absent in *Tox*^{-/-} mice suggesting that *Tox* may function at a point in the DP→CD4⁺CD8^{int} transition that precedes the activity of Gata-3 or THEMIS^{7, 32}. Similar to *Themis*^{-/-} mice, no defect in proximal TCR signaling is observed in *Tox*^{-/-} DP thymocytes indicating that both proteins function at more distal points in the signaling cascade³².

The identification of a specific role for THEMIS in the DP→CD4⁺CD8^{int} transition, together with a large and accumulating list of other nuclear factors involved in this transition, underscores the importance of this stage of thymocyte maturation. The complex array of participating factors is perhaps not unexpected in light of the fact that both thymocyte selection and CD4 versus CD8 lineage choice occur simultaneously during this transition. Additional studies aimed at identifying the function of THEMIS at the cellular

level should aid in elucidating the signaling pathways that regulate the developmental transition of immature DP thymocytes into mature, functional T cells.

Methods

Isolation of the murine E430004N04Rik (Themis) cDNA clones

CD117⁺CD25⁻ and CD117⁻CD25⁺ cells were isolated by magnetic separation from embryonic day 15 fetal thymocytes obtained from C57BL/6 mice. Total RNA was isolated using Trizol (Life Technologies). cDNA synthesis and subtraction were performed using a PCR-Select cDNA subtraction kit (Clontech). The cDNA fragments enriched in CD117⁻CD25⁺ fetal thymocytes were directly ligated into a T/A cloning vector (Invitrogen) and sequenced. For individual clones of interest, enhanced expression in CD117⁻CD25⁺ fetal thymocytes relative to CD117⁺CD25⁻ fetal thymocytes was confirmed by semi-quantitative RT-PCR analysis. The full-length sequence of murine *THEMIS* cDNA was obtained by screening a murine fetal (embryonic day 15.5) thymus cDNA library and 5'RACE analysis of murine total thymus cDNA. Human *Themis* cDNA was cloned by screening a Jurkat cDNA library.

Mice

Themis^{-/-} mice were generated by homologous recombination-mediated gene targeting in 129 strain ES cells using the methods described previously³³ and according to the strategy outlined in Supplementary Fig. 4. The Gata-3 transgene was generated using the hCD2-based transgenic expression vector p29delta2³⁴. H-Y αβTCR transgenic, AND αβTCR transgenic mice, *B2m*^{-/-} mice and *H2Ab1*^{-/-} mice were obtained from Taconic Farms. *Bcl-2* transgenic and *Cd8a*^{-/-} mice were kindly provided by A. Singer (National Cancer Institute). *Th-POK* transgenic mice were previously reported³⁵.

Antibodies and reagents

To prepare anti-THEMIS antibodies, two peptides corresponding to residues 205-218 (CDFSNKW DSTNPFPE) or 596-608 (CKKLPSDESGQDSR) of mouse THEMIS were coupled to KLH and independently injected into New Zealand White rabbits (Covance). Anti-THEMIS antibodies were affinity purified from rabbit serum using a covalently bound antigen column. Sources for antibodies used in this study include: anti-IκBα anti-phospho-ERK(T202/Y204), anti-phospho-LAT(Y136), anti-phospho-ZAP(Y314), anti-phospho-Akt(S473) and anti-phospho-JNK(T183/Y185), Cell Signaling Technology; Anti-phospho-Vav(Y160) and anti-phospho-PLCγ1(Y483), Invitrogen; Anti-Gata-3 (HG3-31), anti-Egr-1 (C-19), anti-PLCγ1 (1249) and anti-histone H1 (FL-219), Santa Cruz Biotechnologies; FoxP3-PE (FKJ-16s), ebioscience; annexin V-FITC, anti-Gata-3 (L50-823)-alexa-488, anti-Bcl-2 (3F11) and anti-Grb2 (81), BD Biosciences; Polyclonal anti-cMyb, Millipore. Rabbit polyclonal anti-Th-POK antibodies were described previously⁷. Rabbit polyclonal anti-TOX was provided by J. Kaye and anti-Runx-1/3 was provided by D. Littman. CD1d tetramers loaded with PBS57 were used to stain NKT cells (NIH tetramer facility). Intracytoplasmic staining was performed using the fixation/permeabilization buffer from e-Bioscience.

Isolation of thymocyte subsets and flow cytometry analysis

CD69⁺ thymocytes were purified by positive selection using anti-FITC magnetic beads and magnetic columns from Miltenyi Biotec. DP and CD4 SP immature thymocytes were purified by negative selection after retention of CD3^{hi}CD25⁺ or CD8⁺CD62L⁺CD25⁺ thymocytes, respectively, on magnetic columns. Thymi and lymph nodes were excised from mice and single-cell suspensions were prepared in FACS buffer (PBS, 0.1% BSA, 0.1% azide). Analysis was performed on a Becton Dickinson Immunocytometry Systems

FACScalibur or LSR with standard CellQuest software. Calcium flux measurements were performed as described³⁶. Statistical analyses were performed using the *t* test function of Microsoft Excel (two-tailed, same variance).

Generation of bone marrow chimeras

Bone marrow cells from congenic (CD45.1⁺) C57BL/6 mice and from *Themis*^{+/-} or *Themis*^{-/-} mice (CD45.2⁺) were mixed at a 1:4 ratio and injected into irradiated (650 rads) CD45.1⁺ C57BL/6 mice. Mice were analyzed 8 weeks after bone marrow transfer.

Subcellular fractionation

10⁷ thymocytes were incubated in 120 µl of hypotonic buffer (Hepes 100 mM, KCl 10 mM, EDTA 1 mM, Na₃VO₄ 2 mM, protease inhibitors tablet (Roche)) for 20 min on ice. After incubation, 1.2 µl of NP40 10% was added. 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 µl of nuclear lysis buffer (Hepes 100 mM, NaCl 400 mM, EDTA 1 mM, Na₃VO₄ 2 mM, protease inhibitors tablet) for 10 min on ice. Lysates were centrifuged at 14000 r.p.m., for 10 min. Supernatants, which contain nuclear extract, were collected.

In vitro DP→CD4+CD8^{int} development assay

The in vitro development assay was performed essentially as described¹⁵. Briefly, purified DP thymocytes were resuspended in RPMI 1640 (supplemented with 50 µM 2-mercaptoethanol and 10% charcoal/dextran treated FBS) and incubated overnight in wells coated with anti-TCRβ (H57) + anti-CD2 (RM2-5). Cells were extensively washed and either analyzed immediately by flow cytometry (stimulatory culture) or incubated for 24 h in the same medium prior to analysis by flow cytometry (recovery culture).

Microarray

CD69⁺ thymocytes from *Themis*^{+/-} and *Themis*^{-/-} littermates (four mice per group) were purified with magnetic beads. RNA was isolated using the RNA purification kit from Ambion. RNA was amplified and biotinylated using Illumina TotalPrepTM RNA Amplification Kit and hybridized to Illumina Sentrix[®] microchips arrays. Microarray data was analyzed using DIANE 6.0, a spreadsheet-based microarray analysis program based on SAS JMP7.0 system. Raw microarray data were subjected to filtering and Z normalization and tested for significant changes as previously described³⁷. The sample quality was analyzed by gene sample z score based hierarchy clustering to exclude possible outliers. The expression of individual genes with Z ratio > 2 and fdr < 0.3 were considered significantly different.

Real-time PCR

For gene expression studies, total cell RNA was isolated using a PicoPure RNA isolation kit (Arcturus). 100 ng of each RNA sample was reverse-transcribed using the SuperScript first-strand synthesis system (Invitrogen) and assayed by RT-PCR. Transcript quantification was performed with a Roche LightCycler 480. Duplicates were run for each sample in a 96-well plate. β-actin was used as the endogenous reference gene. The relative quantification method was used, with the ratio of the mRNA abundance of the gene of interest normalized to the abundance of β-actin mRNA and the average of control thymocyte samples as the calibrator value. The specificity of the products was confirmed based on melting curves and electrophoresis.

TCR stimulation assays

For *in vivo* TCR stimulation, adult MHC class II-deficient (*H2Ab1*^{-/-}) mice were injected intraperitoneally with 30 µg of anti-TCR (H57)/gm body weight and sacrificed four days later for thymocyte analysis. For *in vitro* activation assays, thymocytes from MHC deficient (*B2m*^{-/-} × *H2Ab1*^{-/-}) *Themis*^{+/+} or *Themis*^{-/-} mice, which consisted of >95% DP cells, were activated as described in the text. 10⁷ thymocytes were stimulated at 37°C with pre-formed immune complexes composed of biotinylated anti-CD3 (145-2C11, 5 µg/ml), anti-CD4 (GK1.5, 5 µg/ml) and streptavidin (10 µg/ml). 2×10⁶ cell equivalents were loaded on 4-12% gradient gels (Invitrogen) and analyzed by immunoblot with the indicated antibodies. The SH3 domain array was purchased from Panomics and used according to the manufacturer's recommendations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Structure and expression of E430004N04Rik (THEMIS). **a)** Schematic representation of mouse THEMIS and its human ortholog. NLS, Nuclear Localization Signal; PRR, Proline rich region. Percent identities of the indicated domains are shown. **b)** Northern blot analysis of THEMIS expression in mouse tissues. **c)** Immunoblot analysis of THEMIS expression in thymocytes and peripheral lymphoid cells. IEL, intraepithelial lymphocytes. **d)** Analysis of THEMIS expression in mouse thymocyte subsets by intracellular staining. Bars show THEMIS expression in gated thymocyte populations from *Themis*^{+/+} or *Themis*^{-/-} mice. Δ geo. mean = geometric mean of THEMIS staining in *Themis*^{+/+} thymocytes – geometric mean of THEMIS staining in *Themis*^{-/-} thymocytes. Graphic is representative of three independent experiments.

Figure 2. Development of SP thymocytes is impaired in *Themis*^{−/−} mice. Analysis of *Themis*^{+/−} and *Themis*^{−/−} thymocytes (**a**) and lymph node T cells (**b**) 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 (normalized to 10⁸ cells/thymus) or lymphocyte subsets calculated from six mice per group. (t-test, **P* < 0.05). **c**) CD3 versus CD24 surface staining of gated CD4SP or CD8SP thymocytes. Numbers are percentage of cells within the indicated gate. Bar graphs show ratio of mature (CD24^{lo/−}) to immature (CD24^{hi}) cells calculated from four mice per group. (t-test, **P* < 0.05).

Figure 3.

Positive and negative selection are impaired in *Themis*^{-/-} mice. **a**) Positive selection of thymocytes in *Themis*^{+/-} or *Themis*^{-/-} mice expressing MHC class II-restricted (AND) or MHC class I-restricted (H-Y) TCR transgenes. Two parameter plots show CD4 versus CD8 staining on total thymocytes. Bar graphs show average cell numbers of DP or SP thymocytes calculated from 4-6 mice per group. (t-test, **P* < 0.05). Single parameter histogram plots show CD5 staining on DP thymocytes. **b**) Negative selection of thymocytes in male *Themis*^{+/-} and *Themis*^{-/-} mice expressing the class I-restricted H-Y TCR transgene. Numbers below plots show percent of cells within each quadrant. Bar graphs are average cell numbers of total thymocytes or DP thymocytes calculated from 4-6 mice per group. (t-test, **P* < 0.05).

Figure 4.

The generation of CD4⁺CD8^{int} thymocytes is impaired in *Themis*^{-/-} mice. **a**) Two parameter plots show CD3 versus CD69 surface staining on total thymocytes from *Themis*^{+/-} or *Themis*^{-/-} mice. Bar graphs represent average cell numbers of the indicated thymocyte subsets calculated from 4 mice per group. (t-test, **P* < 0.05). **b**) Flow cytometry analysis of thymocytes from *H2Ab1*^{-/-}*Themis*^{+/-} and *H2Ab1*^{-/-}*Themis*^{-/-} mice. Two parameter plots show CD4 versus CD8 staining on gated CD3^{hi} thymocytes. Bar graphs are average numbers of CD3^{hi}CD4⁺CD8^{int} thymocytes calculated from 4 mice per group. (t-test, **P* < 0.05). **c**) DP thymocytes from *Themis*^{+/-} or *Themis*^{-/-} mice were left unstimulated (medium) or stimulated overnight with anti-TCRβ + anti-CD2. Thymocytes were analyzed immediately by flow cytometry (stimulatory) or washed and incubated in medium without stimulation for an additional 24 h prior to FACS analysis (recovery). Two parameter plots show CD4 versus CD8 staining profiles. Data are representatives of four independent experiments.

a **b****MHC I-KO** **Figure 5.**

Evidence of a signaling defect in *Themis*^{-/-} thymocytes at the CD4⁺CD8^{int} stage. **a)** Comparison of surface expression of CD3, CD5, CD69 and IL-7 receptor on gated CD4⁺CD8^{int} thymocytes from *Themis*^{+/+} and *Themis*^{-/-} mice. **b)** MHC class II-restricted thymocytes are redirected to the CD8 lineage in *Themis*^{-/-} mice. Two parameter plots show CD4 versus CD8 staining of thymocytes or lymph node T cells from *B2m*^{-/-}*Themis*^{+/+} or *B2m*^{-/-}*Themis*^{-/-} mice. Single parameter histograms show CD5 and CD69 surface staining on CD4⁺CD8^{int} thymocytes from *B2m*^{-/-}*Themis*^{+/+} or *B2m*^{-/-}*Themis*^{-/-} mice. Data are representatives of five independent experiments.

Figure 6.

Gata-3 and Th-POK expression are reduced in *Themis*^{-/-} CD69⁺CD4SP thymocytes. **a)** Cell extracts from purified CD69⁺ thymocytes from *Themis*^{+/+} and *Themis*^{-/-} mice were analyzed by immunoblot with antibodies specific for the indicated proteins. **b)** Gata-3 intracellular (IC) staining of the indicated thymocyte subsets from *Themis*^{+/+} and *Themis*^{-/-} mice. **c)** Tox and Th-POK gene expression in purified CD69⁺CD4SP thymocytes from *Themis*^{+/+} and *Themis*^{-/-} mice measured by real-time PCR. All data are representatives of three independent experiments.

Figure 7.

Gata-3 and Th-POK are induced in *Themis*^{-/-} thymocytes by strong TCR signals. **a)** Two parameter plots of thymocytes from *H2Ab1*^{-/-}*Themis*^{+/-} or *H2Ab1*^{-/-}*Themis*^{-/-} mice four days after intraperitoneal injection of PBS or anti-TCR β . **b)** Intracellular staining for Gata-3 and surface staining for CD5 on gated CD4⁺CD8^{int} thymocytes isolated from mice four days after anti-TCR β injection. **c)** Expression of Th-POK in total thymocytes four days after anti-TCR β injection determined by real-time PCR. Data are representative of two independent experiments (three mice for each group).

Figure 8. Forced expression of Th-POK or Gata-3 does not rescue CD4SP development in *Themis*^{-/-} mice. **a)** Flow cytometry analysis of thymocytes and lymph node cells from *Themis*^{+/-} and *Themis*^{-/-} mice with or without the Gata-3 transgene. Data are representative of four independent experiments. **b)** Flow cytometry analysis of thymocytes and lymph node cells from *Themis*^{+/-} and *Themis*^{-/-} mice with or without the Th-POK transgene. Data are representative of three independent experiments.

Figure 9.

Evidence for a survival defect in immature CD8SP thymocytes. **a)** Two parameter dot plots show CD4 versus CD8 staining of total thymocytes or CD24 versus TCR β staining of gated CD8SP TCR^{hi} thymocytes from *Themis*^{+/-} and *Themis*^{-/-} mice with or without the *Bcl-2* transgene. Numbers in plots indicate percentage of cells within each quadrant or gate. Data are representative of three independent experiments. **b)** Bar graphs show numbers of CD8SP, CD4SP and CD4⁺CD8^{int} thymocytes in the indicated mice. NT, non-transgenic. Three mice were analyzed for each group.