THEMIS enhances TCR signaling and enables positive selection by selective inhibition of SHP-1

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Abstract

THEMIS, a T cell specific protein that is highly expressed in $CD4^+CD8^+$ thymocytes, has a crucial role in positive selection and T cell development. THEMIS lacks defined catalytic domains but contains two tandem repeats of a distinctive (CABIT) module of unknown function. Here, we show that THEMIS directly regulated the catalytic activity of the protein tyrosine phosphatase SHP-1. This action was mediated by the CABIT modules, which bound to the SHP-1 phosphatase domain and promoted or stabilized oxidation of the SHP-1 catalytic cysteine, inhibiting SHP-1 tyrosine phosphatase activity. Reduction of SHP-1 alleviated the developmental block in *Themis* $^{-/-}$ thymocytes. Thus, THEMIS facilitates thymocyte positive selection by enhancing the T cell antigen receptor signaling response to low affinity ligands.

Keywords

lymphocyte; T-cell; thymocyte; development; positive selection; tyrosine phosphatase; signal transduction; evolution; CABIT module

INTRODUCTION

T cell development is a continuous process that begins when progenitor cells that originate in the fetal liver or adult bone marrow enter the thymus and are induced to commit to the T

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cell lineage. Thymocytes progress through multiple well-defined maturational steps that for simplicity are grouped into three main stages defined by expression of the CD4 and CD8 co-receptors: double negative (DN), double positive (DP) and CD4 or CD8 single positive (CD4 SP or CD8 SP). Transition of thymocytes through these stages of maturation is dependent upon signals transmitted by numerous cell surface molecules including Notch, cytokine receptors and precursor or mature forms of the T cell antigen receptor (TCR)[1, 2].

All thymocytes are subjected to a selection process at the DP stage based on the affinity of their expressed TCR for self-peptide ligands bound to self-major histocompatibility complex (self-pMHC) that tests TCR functionality and enforces self tolerance[3]. Thymocytes that express TCRs that fail to bind to self-pMHC or that bind with high affinity to self-pMHC are 'non-selected' or 'negatively selected', respectively, and are triggered to undergo apoptotic cell death, whereas thymocytes that express TCRs that bind with low affinity to self-pMHC are 'positively selected' and progress to the CD4 SP or CD8 SP stage[3]. TCR-self-pMHC affinity controls the intensity and duration of the TCR signaling response, which in turn leads to the differential activation of downstream signal transduction pathways and transcriptional responses that dictate cell fate[4].

Thymocyte selection is dependent upon the expression and function of several lineage-restricted effector molecules including the protein tyrosine kinases (PTKs) LCK and ZAP-70, the protein tyrosine phosphatase (PTP) SHP-1, and specialized adaptors such as LAT and SLP-76[5, 6]. THEMIS, a T cell specific protein, was shown to have an important role in thymocyte selection. In the absence of THEMIS, thymocyte development is partially blocked at the DP to SP transition stage resulting in a severe reduction in mature CD4 SP and, to lesser extent, CD8 SP thymocytes and peripheral T cells[7, 8, 9, 10, 11].

THEMIS is the founding member of a group of structurally related proteins that are defined by the presence of one or more copies of a newly described CABIT (cysteine-containing all beta in THEMIS) globular module with a median length of 261 amino acids that contains a conserved core motif (ϕXCX$_{7-26}$$\phi$XLP$\phi$X$_3$GXF; X = any amino acid, ϕ = any hydrophobic residue)[9]. All mammalian THEMIS family members, including THEMIS, THEMIS2, which is restricted to B and myeloid cells, and the more distantly related THEMIS3, which is expressed in the large and small intestine[7], contain two tandem CABIT modules and a C-terminal proline-rich sequence (PRS), but lack a known catalytic domain[9]. THEMIS binds directly to the cytosolic adapter GRB2 and this interaction requires the THEMIS PRS[12, 13]. Mass-spectrometry screens of proteins co-immunoprecipitated with THEMIS identified the protein tyrosine phosphatase SHP-1 as a putative THEMIS interacting protein[14, 15], and it has been suggested that THEMIS functions by regulating SHP-1 activity or its recruitment to LAT[16, 17]. Nevertheless, a specific role for THEMIS in T cell development has not been clearly defined, and it remains unclear if its effect on TCR signaling is primarily activating or inhibitory[14, 15, 16]. A particular challenge has been to identify a function for the CABIT modules that comprise most of the THEMIS protein. The presence of highly-conserved core sequences, and their requirement for THEMIS activity *in vivo*[18] strongly suggests that CABIT modules have an important biological role; however, their distinctiveness from all previously described protein domains indicates that they may perform a unique cellular function[9].

In this study, we identify a biological function for the CABIT modules that clarifies the role of THEMIS in T cell development. We show that the THEMIS CABIT modules bind directly to the SHP-1 PTP domain and inhibit SHP-1 PTP activity by promoting or stabilizing oxidation of the catalytic cysteine. This activity, coupled with the stage specific regulation of THEMIS during T cell development[7, 8, 9, 11], provides an explanation for the unusual sensitivity of DP thymocytes to TCR stimulation[19], a property that is essential for positive selection.

RESULTS

THEMIS binds to the SHP-1 PTP domain

To determine if THEMIS binds directly to SHP-1, we first performed cell-free *in vitro* protein binding assays. THEMIS bound to a Glutathione-S-transferase (GST)-SHP-1 fusion protein in the absence of GRB2, although the THEMIS:SHP-1 interaction was enhanced by GRB2 (Fig. 1a). In lysates from HEK-293 cells co-transfected with plasmids encoding SHP-1 or a THEMIS protein lacking the GRB2 binding PRS sequence (THEMIS-1-493), SHP-1 was co-immunoprecipitated with THEMIS-1-493 (Fig. 1b). GRB2-independent association of THEMIS and SHP-1 was also detected by co-immunoprecipitation of THEMIS with SHP-1 from lysates of GRB2-deficient total thymocytes (Fig. 1c).

SHP-2, another dual SH2 PTP that is closely related to SHP-1, but not to two other class I PTPs that are expressed in thymocytes (PTPN1, PTPN7), bound to THEMIS when co-expressed in HEK-293 cells (Supplementary Fig. 1a,b). To localize the sequences within THEMIS that mediate binding to SHP-1, we next performed co-immunoprecipitation experiments in HEK-293 cells transfected with plasmids encoding SHP-1 and different THEMIS truncations. SHP-1 co-immunoprecipitated with a THEMIS protein that contains only the CABIT1 and CABIT2 modules (THEMIS-1-493), and to a lesser extent, with truncated THEMIS proteins containing only the CABIT1 (THEMIS-1-260) or CABIT2 (THEMIS-260-493) module (Fig. 1b). In similar co-transfection experiments, a protein containing only the PTP domain of SHP-1 co-immunoprecipitated with THEMIS-1-493 and with THEMIS-1-260 (Fig. 1d). Also, a purified SHP-1 protein lacking both SH2 domains but containing the PTP domain (GST- SH2-SHP-1) bound to THEMIS-1-493 *in vitro* (Fig. 1e and Supplementary Fig. 1c,d). Together, these results demonstrated that the THEMIS CABIT modules interact directly with the PTP domain of SHP-1.

THEMIS CABIT modules inhibit SHP-1 PTP activity

To determine if THEMIS directly regulates the PTP activity of SHP-1, we used an *in vitro* assay to detect phosphate release from a tyrosine phosphorylated peptide. The PTP activity of SHP-1 was reduced in the presence of THEMIS and the reduction correlated with the concentration of THEMIS protein (Fig. 2a). SHP-1 PTP activity was not inhibited by GRB2, which also binds to SHP-1 demonstrating that the inhibitory effect was specific to THEMIS; however, inhibition of SHP-1 was slightly greater when THEMIS and GRB2 were added together (Fig. 2b). In a similar assay, the PTP activity of SHP-2 was slightly reduced by THEMIS, whereas THEMIS did not inhibit the PTP activity of PTPN1 and PTPN7 (Fig. 2c). THEMIS1-493 was nearly as effective as full-length THEMIS at inhibiting the PTP activity

of SHP-1 (Fig. 2d), indicating that the CABIT modules are responsible for the inhibitory function of THEMIS. THEMIS-1-260 also inhibited the PTP activity of SHP-1, though not as effectively as THEMIS-1-493 which contains both CABIT modules (Fig. 2d), indicating that a single CABIT module contains the sequences necessary for regulating SHP-1 PTP activity but both CABIT modules are required for full inhibition. THEMIS2, which rescued the developmental block in *Themis*^{−/−} thymocytes when transgenically expressed in thymocytes, and therefore could substitute for THEMIS *in vivo*[13], inhibited the PTP activity of SHP-1 (Fig. 2e), and to a lesser extent SHP-2, but not PTPN1 or PTPN7 in *in vitro* tyrosine phosphatase assays (Fig. 2c).

All mammalian CABIT modules contain a conserved cysteine within the $\phi X \underline{C} X_{7-26} \phi XLP \phi X_3 GXF$ core motif[9]. To determine the role of the cysteine residue for THEMIS regulatory activity, we introduced cysteine to alanine point mutations in both the CABIT1 (C153) and CABIT2 (C413) modules of THEMIS (THEMIS-C-A). THEMIS-C-A was co-immunoprecipitated SHP-1 in lysates from transfected HEK-293 cells (Supplementary Fig. 1e) and inhibited SHP-1 PTP activity in an *in vitro* tyrosine phosphatase assay (Fig. 2e), indicating that the cysteine residues in the CABIT domains are not essential for regulating the PTP activity of SHP-1. This is consistent with previous observations that retrovirally encoded THEMIS-C-A can rescue the DP to SP developmental block in *Themis*^{−/−} thymocytes[20]. These results demonstrated that the THEMIS CABIT modules directly inhibit SHP-1 PTP activity and that the conserved core cysteine is not required for this function.

Reduction of SHP-1 rescues T cell development in *Themis*^{−/−} mice

We next determined if inhibition of SHP-1 PTP activity can reverse the developmental block in *Themis*^{−/−} thymocytes in an *in vitro* differentiation assay[21]. Overnight culture of immature (TCR^{lo}) DP thymocytes with plate-bound CD3+CD2 antibodies followed by a 24 h rest without stimulation induces their progression to the CD4^{+}CD8^{lo} stage[21], replicating the initial stages of positive selection *in vivo*. In contrast to wild-type DP thymocytes, *Themis*^{−/−} DP thymocytes exhibit impaired ability to transition to the CD4^{+}CD8^{lo} stage (Fig. 3)[7]. The block in development of *Themis*^{−/−} DP thymocytes to the CD4^{+}CD8^{lo} stage was significantly alleviated when the *in vitro* assay was performed in the presence of the selective SHP-1 inhibitor sodium stibogluconate (SSG)[22] (Fig. 3), indicating that increased SHP-1 PTP activity contributes to the developmental defect in *Themis*^{−/−} thymocytes.

To determine if the developmental block in *Themis*^{−/−} thymocytes could be rescued by a reduction in SHP-1 protein expression, we generated *Themis*^{−/−} *Ptpn6*^{fl/fl} *Cd4-Cre* mice, in which T cell lineage-specific deletion of the gene encoding SHP-1 (*Ptpn6*) occurs primarily in DP thymocytes[23]. Thymocytes from *Themis*^{−/−} *Ptpn6*^{+/+}*Cd4-Cre* control mice exhibited normal maturation up to the DP stage, but a marked reduction in CD4 SP and to lesser extent CD8 SP thymocytes and peripheral T cells (Fig. 4a,b), which is similar to the developmental phenotype of *Themis*^{−/−} mice[7]. SHP-1 protein in *Themis*^{−/−} *Ptpn6*^{fl/fl} *Cd4-Cre* total thymocytes was reduced to approximately 25% of that observed in *Themis*^{−/−} *Ptpn6*^{+/+}*Cd4-Cre* total thymocytes as assessed by densitometry (Fig. 4c). This reduction in SHP-1 expression alleviated the developmental block, as evinced by a significant increase in the

percentage and number of mature TCR[hi] CD4 SP and CD8 SP thymocytes and peripheral T cells in *Themis^{−/−} Ptpn6^{fl/fl} Cd4-Cre* mice compared to *Themis^{−/−} Ptpn6^{+/+} Cd4-Cre* controls (Fig. 4a,b). In contrast to *Themis^{−/−} Ptpn6^{+/+} Cd4-Cre* mice, which contained a high percentage of peripheral T cells with a CD62L^{lo/−} CD44^{hi} memory T cell phenotype as a result of lymphopenia-induced expansion[7], the percentage of CD62L^{lo/−} CD44^{hi} T cells was similar in *Themis^{+/+}Ptpn6^{+/+} Cd4-Cre* and *Themis^{−/−}Ptpn6^{fl/fl} Cd4-Cre* mice (Fig. 4a). Deletion of *Ptpn6* at an earlier stage of development (predominantly at the DN stage) using an *LCK*–Cre transgene[23] resulted in a near complete absence of SHP-1 protein in total thymocytes and also significantly alleviated the developmental block *Themis^{−/−} Ptpn6^{fl/fl} LCK-Cre* mice compared to *Themis^{−/−} Ptpn6^{+/+} LCK-Cre* control mice (Supplementary Fig. 2). Together, these results indicated that the developmental defect in *Themis^{−/−}* thymocytes is caused by enhanced SHP-1 PTP activity.

THEMIS regulates active site oxidation of SHP-1

All classical PTPs, including SHP-1 and SHP-2, contain a conserved active site cysteine with an unusually low pKa that catalyzes the removal of phosphate from phosphorylated tyrosines[6]. However, the catalytically active deprotonated (S[−]) thiolate state of the active site cysteine is highly susceptible to oxidation by intracellular reactive oxygen species (ROS) which inactivates the PTP[6]. To test if binding of THEMIS to SHP-1 directly regulates the redox state of the active site cysteine, we added pervanadate, a pan-tyrosine phosphatase inhibitor that irreversibly oxidizes PTP active site cysteine residues to the sulfonic acid (S-O_3H) form[24], to cell-free suspensions of GST-SHP-1 in the presence or absence of THEMIS. Oxidized SHP-1 was detected by immunoblotting with a monoclonal antibody specific for sulfonylated PTP active site cysteines[25]. Oxidation of SHP-1 by pervanadate was enhanced in the presence of THEMIS, and this effect was most evident at pervanadate concentrations that resulted in sub-maximal SHP-1 oxidation (Fig. 5a and Supplementary Fig. 3a). THEMIS also increased the susceptibility of SHP-1 to oxidation by pervanadate when THEMIS and SHP-1 were co-transfected into HEK-293 cells (Fig. 5b and Supplementary Fig. 3b).

To evaluate the redox status of SHP-1 in *Themis^{−/−}* thymocytes, we labeled catalytically active SHP-1 at the time of cell lysis by addition of iodoacetyl-PEG-biotin (IAP-bio), which immediately and irreversibly binds to reduced de-protonated (-S[−]) cysteine thiols[25]. Catalytically active SHP-1 was modestly but consistently increased in *Themis^{−/−}* total thymocytes and was decreased in THEMIS transgenic (Tg)[14] total thymocytes compared to control *Themis^{+/+}* total thymocytes (Fig. 5c). Lysis of thymocytes in the absence of PTP inhibitors results in the rapid oxidation and inactivation of SHP-1 (Supplementary Fig. 3c,d); consequently, we were unable to accurately evaluate the PTP activity of SHP-1 protein immunoprecipitated from thymocyte lysates by tyrosine phosphatase assay. However, SHP-1 was much less susceptible to oxidation by pervanadate in *Themis^{−/−}* total thymocytes compared to *Themis^{+/+}* total thymocytes (Fig. 5d and Supplementary Fig. 3e). The susceptibility of SHP-1 to oxidation by pervanadate was restored in *Themis^{−/−}* Themis2-Tg thymocytes[13] (Fig. 5e), demonstrating shared function of THEMIS and THEMIS2. T cell and B cell activation results in the production of ROS, predominantly H_2O_2, and this effect has been shown to positively regulate both TCR and BCR signaling and effector responses

through oxidative inhibition of SHP-1[26, 27, 28]. In the presence of THEMIS, active site oxidation of SHP-1 by H_2O_2, assessed by immunoblotting with sulfonylated PTP active site antibody, was markedly increased (Fig. 5f), indicating that THEMIS regulates the redox state of SHP-1 in response to physiological ROS. Together, these results demonstrated that THEMIS inhibits SHP-1 PTP activity by promoting or stabilizing ROS-mediated oxidation of the SHP-1 active site cysteine.

Tyrosine phosphorylation of SHP-1 does not correspond with PTP activity

We found that phosphorylation of SHP-1, which occurs at two C-terminal tyrosine residues (Y536 and Y564), was reduced in $Themis^{-/-}$ total thymocytes compared to $Themis^{+/+}$ total thymocytes (Fig. 6a,b), confirming previous results[16]. The amount of tyrosine phosphorylated SHP-1 (p-SHP-1) in total thymocytes correlated with the expression of THEMIS protein and p-SHP-1 levels were restored in $Themis^{-/-}$ thymocytes by expression of a THEMIS2 transgene[13] (Figs. 6a-c). The reduced p-SHP-1 in $Themis^{-/-}$ thymocytes was previously interpreted as evidence of reduced SHP-1 catalytic activity[16]; however, SHP-1 phosphorylation is not required for its PTP catalytic activity, and its physiological relevance has not been established[6, 29]. In un-stimulated $Themis^{-/-}$ total thymocytes, the amount of catalytically active SHP-1 was increased not reduced compared to $Themis^{+/+}$ total thymocytes (Fig. 5c,f). Because SHP-1 is a target of SHP-1 phosphatase[6, 29, 30], we reasoned that the reduction in p-SHP-1 in $Themis^{-/-}$ thymocytes was secondary to increased auto- or trans-dephosphorylation by SHP-1. Indeed, treatment of both $Themis^{+/+}$ and $Themis^{-/-}$ total thymocytes with PV or H_2O_2, which inhibit SHP-1 activity, led to an increase in p-SHP-1 compared to untreated controls (Fig. 6d,e). Together, these results demonstrate that SHP-1 phosphorylation status does not predict SHP-1 PTP catalytic activity, and suggest that the reduced p-SHP-1 in $Themis^{-/-}$ thymocytes is secondary to enhanced auto- or trans-dephosphorylation by SHP-1.

TCR signaling defects in $Themis^{-/-}$ thymocytes in the presence of ROS

In contrast to mature T cells where TCR engagement induces production of ROS[31, 32, 33], TCR stimulation of thymocytes fails to elicit ROS (Supplementary Fig. 4a)[34]. However, ROS are induced in thymocytes by stimulation with the lectin concanavalin A (ConA)[34], which engages multiple cell surface molecules in addition to the TCR. Tyrosine (Y319) phosphorylation of the PTK ZAP-70, a known target of SHP-1[6, 35] was reduced in $Themis^{-/-}$ total thymocytes compared to $Themis^{+/+}$ total thymocytes following stimulation with ConA (Fig. 7a), whereas no difference in tyrosine phosphorylated ZAP-70 (p-ZAP-70) was observed in $Themis^{-/-}$ and $Themis^{+/+}$ total thymocytes following stimulation with CD3+CD4 antibodies (Fig. 7b,c). However, when H_2O_2 was added at the time of CD3+CD4 antibody stimulation, p-ZAP-70 induction was reduced in $Themis^{-/-}$ total thymocytes compared to $Themis^{+/+}$ total thymocytes (Fig. 7b). In vitro cell culture promotes ROS production due to high oxygen tension as well as pro-oxidant metabolic and media effects[36]. Freshly harvested $Themis^{-/-}$ total thymocytes exhibited no clear defects in proximal TCR signaling responses compared to $Themis^{+/+}$ total thymocytes (Fig. 7c). However, after in vitro culture for 6 h, induction of p-ZAP-70, as well as induction of tyrosine (Y394) phosphorylated LCK, another putative SHP-1 target[37] was reduced in $Themis^{-/-}$ total thymocytes compared to $Themis^{+/+}$ total thymocytes in response to either CD3+CD4

antibody stimulation (Fig. 7c,d) or to peptide-APC stimulation (Supplementary Fig. 4b). *Themis*$^{-/-}$ total thymocytes also exhibited reduced induction of p-LCK and p-ZAP-70 in response to H_2O_2 treatment alone compared to identically treated *Themis*$^{+/+}$ total thymocytes (Fig. 7e). De-phosphorylation of ZAP-70, or the related B cell PTK SYK, by SHP-1 was inhibited by THEMIS in transfected HEK-293 cells cultured under conditions where ROS are constitutively produced[36] (Fig. 7f,g). Themis-mediated inhibition of SHP-1 PTP activity in transfected cells (Supplementary Fig. 5a), or *in vitro* (Supplementary Fig. 5b), was attenuated by addition of the ROS scavenger N-Acetyl-L-cysteine (NAC), indicating that the redox regulation of SHP-1 by THEMIS is dependent upon ROS. Collectively, these results establish a positive role for THEMIS in proximal TCR signaling by promoting or stabilizing SHP-1 oxidation by ROS.

DISCUSSION

Here, we show that a critical function of THEMIS during T cell development is to negatively regulate the activity of the protein tyrosine phosphatase SHP-1 in DP thymocytes, thereby enhancing the TCR signaling response to low affinity self-pMHC and enabling positive selection. THEMIS promotes or stabilizes the oxidation of the SHP-1 catalytic cysteine, inhibiting PTP activity, and this regulatory activity is conferred by the CABIT modules that bind directly to the SHP-1 PTP domain.

While it remains to be determined how the CABIT modules regulate oxidation of SHP-1, several plausible mechanisms can be suggested based on their inferred structure together with what is already known about the structure and redox regulation of SHP-1. CABIT modules, which are comprised of multiple SH3-like β-barrel domains[9], are likely to form an extensive protein-binding globular interface that specifically recognizes the SHP-1 PTP domain. The catalytic cysteine of all classical PTPs is housed inside a pocket with an aperture that only allows the entry of the phosphate moiety on tyrosines[38]. Thus, binding of the CABIT modules may prevent access of the oxidized SHP-1 catalytic cysteine to reducing agents such as glutathione in the bulk solvent or to cytosolic redox regulatory proteins[39]. Alternatively, the CABIT modules might stabilize the SHP-1 PTP domain in an unfolded state, exposing the catalytic cysteine to oxidation by ROS or preventing reactivation of the oxidized catalytic cysteine by inhibiting intra-molecular relay of sulfenic acid to the two regulatory cysteines (C329 and C363) in SHP-1[40, 41]. It is important to note that our *in vitro* PTP inhibition data suggest that the inhibitory effect of THEMIS cannot be explained solely by redox regulation. Thus, binding of the CABIT modules also likely blocks access of the catalytic cysteine to phosphotyrosine ligands, a mechanism of inhibition that is not mutually exclusive with redox regulation.

Although THEMIS CABIT modules can bind directly to SHP-1, experimental data strongly suggest that the interaction of THEMIS with GRB2 is important for its *in vivo* function[12, 18, 20]. Co-binding of SHP-1 and THEMIS to GRB2 brings these proteins into close proximity and may facilitate and stabilize their direct interaction. In addition, following TCR engagement, GRB2, via its SH2 domain, recruits SHP-1 to tyrosine phosphorylated ligands at the cell membrane including LAT and CD28[42]. Thus, binding to GRB2 ensures that THEMIS is positioned to impact the activity of the cellular portion of

SHP-1 that is presumably the most relevant to TCR signaling. Finally, GRB2 may also be required to position SHP-1 and THEMIS near sites of ROS production by NADPH oxidases at the cell membrane.

It was recently proposed that THEMIS enhances the activity of SHP-1, either directly or by assisting in its recruitment to LAT, thereby acting to dampen TCR signaling in DP thymocytes[16]. According to that model, SHP-1 PTP activity is reduced in Themis$^{-/-}$ DP thymocytes and TCR engagement by low-affinity ligands that normally promote positive selection results in the transduction of enhanced signaling responses that trigger negative selection[16]. Our results do not support this model. If the thymocyte maturation defect in *Themis*$^{-/-}$ mice was caused by reduced SHP-1 activity, reduction of SHP-1 or inhibition of SHP-1 PTP activity should not have rescued, and might possibly have exacerbated, the block in T cell development in *Themis*$^{-/-}$ mice. Instead, inhibition of SHP-1 PTP activity or reduction of SHP-1 protein in DP thymocytes alleviated the developmental block imposed by THEMIS deficiency, identifying enhanced SHP-1 PTP activity as the underlying cause of the maturational defect in *Themis*$^{-/-}$ thymocytes. It is also significant that T cell development is not rescued in either *Themis*$^{-/-}$*Bim*$^{-/-}$[14] or *Themis*$^{-/-}$*Bcl2-tg* mice[7], indicating that the developmental block in *Themis*$^{-/-}$ thymocytes is not secondary to increased negative selection as previously speculated[16].

It is well established that DP thymocytes are more sensitive to TCR stimulation than mature T cells[19]. Although the mechanism(s) underlying this sensitivity have remained unclear, the enhanced signaling capability of DP thymocytes is thought to be especially important for positive selection, which is mediated by signals generated from low affinity TCR-self-pMHC interactions in the thymus. Our results, together with the profound block in positive selection exhibited by *Themis*$^{-/-}$ thymocytes, suggest that THEMIS may be responsible for the selective sensitivity of DP thymocytes to TCR engagement. Consistent with this, THEMIS is highly expressed in DP thymocytes but is down-regulated as thymocytes transition to the SP stage and become less responsive to low affinity self-ligands[7], a property that is necessary for the prevention of autoimmunity. Thus, stage-specific regulation of THEMIS represents a mechanism for transiently and selectively attenuating SHP-1 activity in DP thymocytes to enable positive selection while preserving the SHP-1-mediated inhibitory pathway for limiting mature T cell responsiveness.

Our results suggest that in addition to SHP-1, THEMIS may also regulate the activity of the closely related class I SH2 domain-PTP, SHP-2. THEMIS binds to SHP-2 and SHP-2 PTP activity was attenuated by THEMIS (albeit only mildly in *in vitro* assays). If THEMIS does have an inhibitory effect on SHP-2 in thymocytes, this may help to explain the observation that under certain stimulatory conditions, ERK and calcium signaling responses to TCR engagement were enhanced in *Themis*$^{-/-}$ thymocytes[16]. Unlike SHP-1, which is thought to have an exclusively inhibitory role in signal transduction, SHP-2 positively regulates ERK and calcium-NFAT mediated signaling[6, 43, 44]. However, it is notable that in contrast to the rescue observed in *Themis*$^{-/-}$ *Ptpn6*$^{-/-}$ mice, deletion of *Ptpn11* which encodes SHP-2, did not alleviate the block in positive selection in *Themis*$^{-/-}$ mice (data not shown), indicating that the primary function of THEMIS in thymocytes is to regulate the catalytic activity of SHP-1.

Our results also identify an important role for ROS in thymocyte selection. Previous data suggest that ROS may play a role in positive selection and SP thymocyte maturation, but this mechanism has not been extensively investigated[45, 46]. ROS, generated following T cell activation by the cell membrane NADPH oxidase NOX2 or by mitochondria as a result of 'metabolic reprogramming', or locally produced by macrophages at sites of inflammation, have been shown to positively regulate mature T cell activation and effector responses, in part by inhibiting SHP-1[47]. The origin of ROS production in the thymus remains to be elucidated and could include both intrinsic (thymocyte-derived) and extrinsic (cortical epithelial, dendritic cell or macrophage) sources. The defects in TCR signaling in *Themis*−/− thymocytes, which at least in part stem from reduced oxidative inactivation of SHP-1 by ROS, are most clearly revealed under conditions where ROS are generated or present during TCR-co-receptor engagement. This provides an explanation for the relatively mild signaling defects and the contradictory results reported from studies where activation of *Themis*−/− thymocyte was performed under conditions where ROS are not present or are not produced[7, 8, 14, 16].

Analysis of a comprehensive collection of over 100 species across the eukaryotic tree revealed that the CABIT module is found only in metazoa and that its emergence correlates with the expansion of the phosphotyrosine signaling network (ref.[9] and data not shown). Together with the biochemical function established for THEMIS in the present study, this suggests that the CABIT module evolved in metazoa as a mechanism to regulate phosphotyrosine signaling. The sequence diversity exhibited by CABIT modules outside of the core sequence raises the interesting possibility that different CABIT modules may have evolved to interact with distinct PTPs. This, combined with organ or developmentally restricted expression of CABIT proteins such as exhibited by THEMIS could represent a novel mechanism for selective regulation of PTK-PTP signaling responses in particular cellular contexts or during specific stages of maturation.

DATA AVAILABILITY

The authors declare that the data supporting the findings of this study are available within the paper and its supplementary information files.

ONLINE METHODS

Mice

Themis−/− mice[7] and *Themis* transgenic mice[13] were generated as described. Ptpn6[flox/flox] mice[48] were obtained from Markus Muschen (UCSF). *Grb2*[flox/flox] mice[49] were obtained from Hua Gu (McGill University). *LCK-Cre* and *CD4-Cre*[23] transgenic mice were obtained from Taconic. All animal experiments were performed according to ACUC approved protocols (ASP# 15-020; PEL).

Antibodies and reagents

Stimulation: Biotin-anti-CD3 (553060), Biotin-anti-CD4 (553728), and Biotin-anti-CD28 (553296) were from BD Biosciences. Immunoprecipitation: anti-Flag (F1804) Sigma Aldrich; anti-SHP-1 (SC287), anti-SHP-2 (SC280) Santa Cruz. Western blotting: anti-pTyr

(05321), anti-GST (06332), anti-ERK (06182), anti-SHP-1 (06117) EMD Millipore; anti-pTyr564-SHP-1 (8849), anti-pTyr416-Src (2101) Cell signaling Technology; anti-pTyr319-ZAP-70 (612574), anti-GRB2 (610111) BD Biosciences; anti-LCK (SC433), anti-ZAP-70 (SC574), anti-pERK (SC7383), anti-HA tag (SC805) Santa Cruz; anti-PTPN7 (ab118978) Abcam; anti-Myc tag (M0473) MBL International; anti-SHP-1 (MS1190) Thermo Fisher; anti-pTyr536-SHP-1(SP1571) ECM Biosciences; anti-actin (A5441) Sigma Aldrich; anti-pPLC-γ1 (44-696G) Biosource;, anti-Oxidized PTP active site mAb (MAB2844) R&D Systems. Rabbit polyclonal antisera to THEMIS[7] and THEMIS2[13] have been described. Streptavidin-HRP conjugate was purchased from Sigma Aldrich. Streptavidin was purchased from Southern Biotechnology.

Plasmids and constructs

The constructs for FLAG-tagged THEMIS and deletion mutants were subcloned into pFLAG-CMV2 vector by PCR with THEMIS-eGFP plasmid[9]. GST-THEMIS was subcloned into pEBG vector by PCR. THEMIS (C153A and C413A) was generated by site-directed mutagenesis with the Quik Change Kit (Stratagene). FLAG-THEMIS1-493 was described[13]. FLAG-tagged THEMIS2 was subcloned into pFLAG-CMV2 vector by PCR with mouse cDNA for THEMIS2 from ImaGene. SHP-1 cDNA was obtained from Dr Axel Ullrich (Max-Plank Institute, Germany). SHP-1 deletion mutants were subcloned into pCDNA3 vector by PCR from *Ptpn6* wild type cDNA. *Ptpn7* was provided by Dr Lutz Tautz (Sanford-Burnham Medical Research Institute). Plasmid encoding HA tagged PTPN1 was a gift from Dr Jonathan Chernoff (Fox Chase Cancer Center).

Immunoprecipitation and western blot analysis

Thymocytes were stimulated with anti-CD3 biotin plus anti-CD4 biotin followed by cross-linking with streptavidin. Cells were then washed in ice-cold PBS and unless stated otherwise lysed in Standard lysis buffer [1% Nonidet P-40, 10 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EGTA, 50 mM β-glycerophosphate, 2 mM Na_3VO_4, 10 mM NaF, and protease inhibitors (Roche)]. Immunoprecipitations and western blotting were performed as described[14].

Transient Transfections

HEK-293 cells were cultured in DMEM supplemented with 10% (vol/vol) FBS and 2 mM glutamine, plus penicillin and streptomycin (100 U/ml each). 1×10^6 cells were co-transfected with the appropriate plasmid using Lipofectamine 2000 (Thermo Scientific). For pervanadate treatment, cells were first serum starved for16 h after transfection.

GST pull down assays

GST-SHP-1 protein was purchased from Abcam. His tagged THEMIS protein was purified by Ni-NTA column from the transformed E. coli bacterial strain BL21DE3. GST fusion proteins were incubated with the indicated His tagged proteins in GST binding buffer: [30 mM HEPES (pH 7), 100 mM NaCl, 1% Triton X-100, 1 mM EGTA, 1 mg/ml BSA, 1 mM Na_3VO_4, 10 mM NaF, and protease inhibitors] for 30 min on ice and then glutathione-

Sepharose (GE Healthcare) was added. After incubation for 30 min at 4°C, this mixture was washed three times with GST wash buffer: [0.2% Triton X-100, 1 mM Na_3VO_4 in 1X PBS].

In vitro PTP assay

GST-SHP-1, GST-PTPN1, and GST-PTPN7 fusion proteins were purchased from Abcam. GST-SHP-2 protein was purified with glutathione-Sepharose columns from the transformed E. coli bacterial strain BL21DE3. Purified PTP proteins were incubated with or without His-THEMIS (or variant), His-THEMIS2 and/or GRB2 proteins at a 1:5 molar ratio for 10 min on ice. PTP substrate peptide (RRLIEDAEpYAARG) was added at a concentration of 0.2 mM in phosphatase assay buffer (20-180, EMD Millipore) and incubated for 30 min at room temperature. Released phosphate was detected by addition of malachite green (17-125, EMD Millipore) and quantitated from a standard curve. N-Acetyl-L-cysteine (NAC) was obtained from Sigma (Cat# A9165).

Detection of reduced (catalytically active) SHP-1

Reduced SHP-1 was detected by direct labeling of cell lysates with Iodoacetyl PEG-biotin (21334, Thermo Scientific). Cells were lysed in degassed Oxidation lysis buffer: [50 mM Tris (pH 7.5), 100 mM NaCl, 0.1% SDS, 0.5% Sodium Deoxycholate, 0.5% NP-40, 0.5% Triton X-100, 50 mM NaF, 1 mM PMSF, 0.4 mM Iodoacetyl PEG-biotin, 100 uM DTPA, 200 U/ml catalase, and protease inhibitors]. Lysates were immunoprecipitated with anti-SHP-1 overnight. Protein G-Sepharose was added and lysates were rotated for 1 h at 4°C. Beads were washed three times with Oxidation wash buffer: [50 mM Tris (pH 7.5), 100 mM NaCl, 0.5% NP-40, 0.5% Triton X-100, 50 mM NaF]. Proteins were eluted with SDS loading buffer, separated on SDS-PAGE then transferred to PVDF membranes. Blots were probed with Streptavidin-HRP.

Analysis of SHP-1 oxidation after stimulation with pervanadate or H_2O_2.

Thymocytes or HEK293 cells were stimulated with pervanadate for 10 min or H_2O_2 for 5 min at room temperature. Pervanadate (1 mM stock) was prepared with 1 mM Na_3VO_4 mixed with 5 mM H_2O_2. H_2O_2 (final concentration) was 0.5-5 mM as noted for individual experiments. Following treatment, cells were washed with degassed 1XPBS and lysed in degassed Standard lysis buffer including 10 mM iodoacetamide and 10 mM NEM. SHP-1 oxidation immunoblotting was performed with anti-Oxidized PTP active site antibody.

In vitro oxidation of SHP-1

GST-SHP-1 fusion protein was incubated with glutathione-Sepharose for 30 min in degassed GST binding buffer minus Na_3VO_4 and washed with degassed 1X GST wash buffer minus Na_3VO_4. Beads were incubated with or without His-THEMIS fusion protein in phosphatase assay buffer for 10 min on ice and then pervanadate was added and protein solution was incubated for 10 min at room temperature. Reactions were washed with degassed GST wash buffer minus Na_3VO_4. SHP-1 oxidation was visualized by immunoblotting with anti-Oxidized PTP active site antibody.

In vitro thymocyte differentiation assay

The *in vitro* thymocyte differentiation assay was performed as described[21]. Briefly, DP thymocytes purified by magnetic bead enrichment (Miltenyi) 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 (H57-597) + 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). Sodium stibogluconate (CAS16037-91-5) was from EMD Millipore.

Statistics

For PTP assays and cell (thymocyte and lymphocyte) counts, significance was calculated by t-test, 2-tailed, type 2 (unpaired equal variance).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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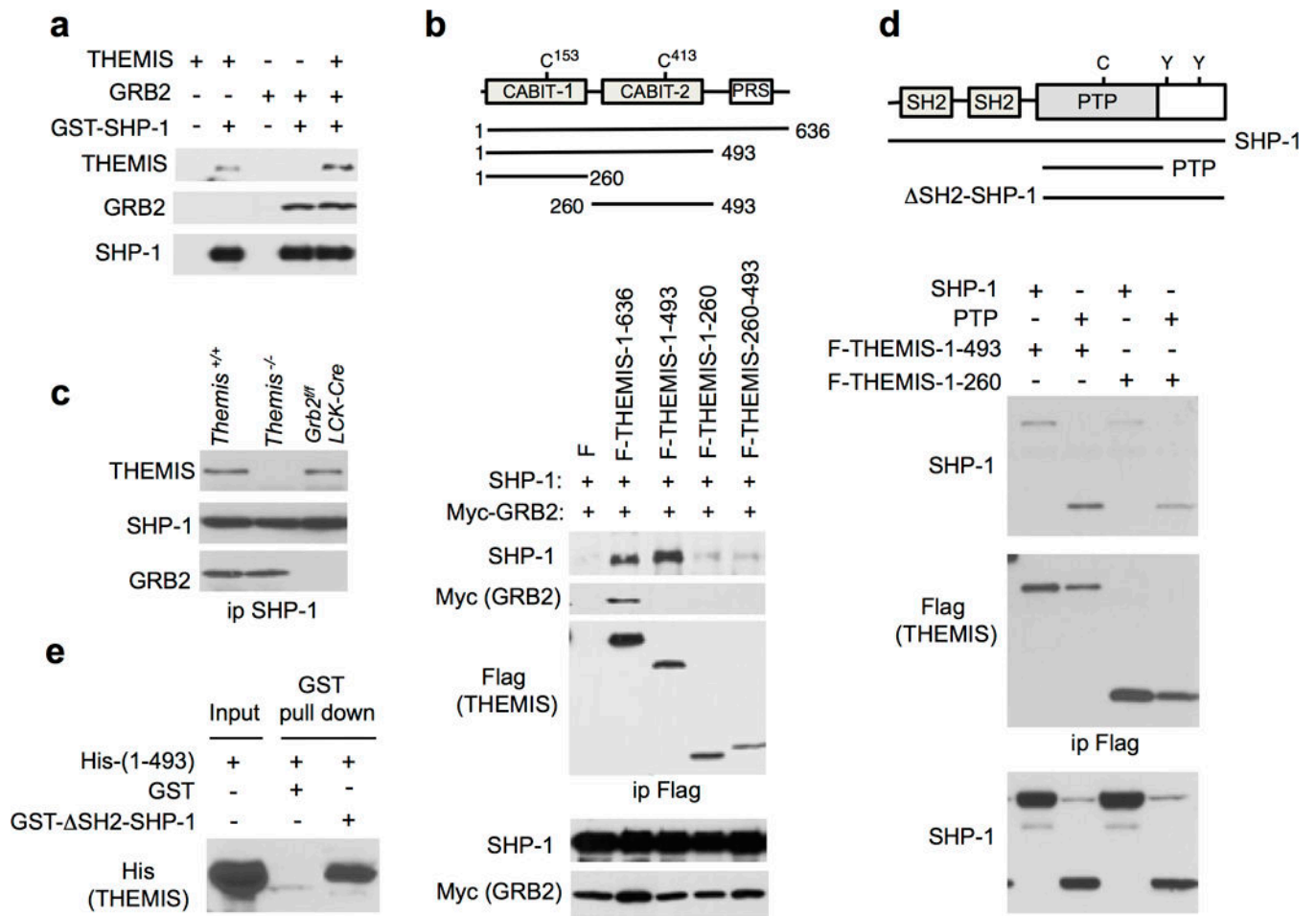
Figure 1. THEMIS binds directly to SHP-1

a, *in vitro* GST-SHP-1 pull down assay showing binding of THEMIS to SHP-1 in the presence or absence of GRB2. **b,** Co-immunoprecipitation experiments in co-transfected HEK-293 cells showing binding of THEMIS CABIT modules to SHP-1 independent of GRB2. Upper panel, schematic of constructs used for transfection. Lower panels, HEK-293 cells were transfected with Flag (F) parent plasmid or plasmids encoding the indicated Flag-tagged THEMIS constructs plus plasmids encoding SHP-1 and Myc-tagged GRB2. Blots are cell lysates (bottom) or anti-Flag immunoprecipitated proteins (top). **c,** Co-immunoprecipitation of THEMIS and SHP-1 from lysates of total thymocytes from the indicated mice. **d,** Co-immunoprecipitation experiments in co-transfected HEK-293 cells showing binding of the THEMIS CABIT modules to the SHP-1 PTP domain. Upper panel, schematic of constructs used for transfection. Lower panels, HEK-293 cells were transfected with the indicated Flag-tagged THEMIS constructs plus plasmids encoding SHP-1 or the SHP-1 PTP domain (PTP). Blots are cell lysates (bottom) or anti-Flag immunoprecipitated proteins (top). **e,** *In vitro* GST-SHP-1 pull down assay showing binding of the THEMIS CABIT modules to the SHP-1 PTP domain. Schematic of ΔSH2-SHP-1 is shown in Fig. 1d. Data shown in **a-e** are representative of three experiments each.
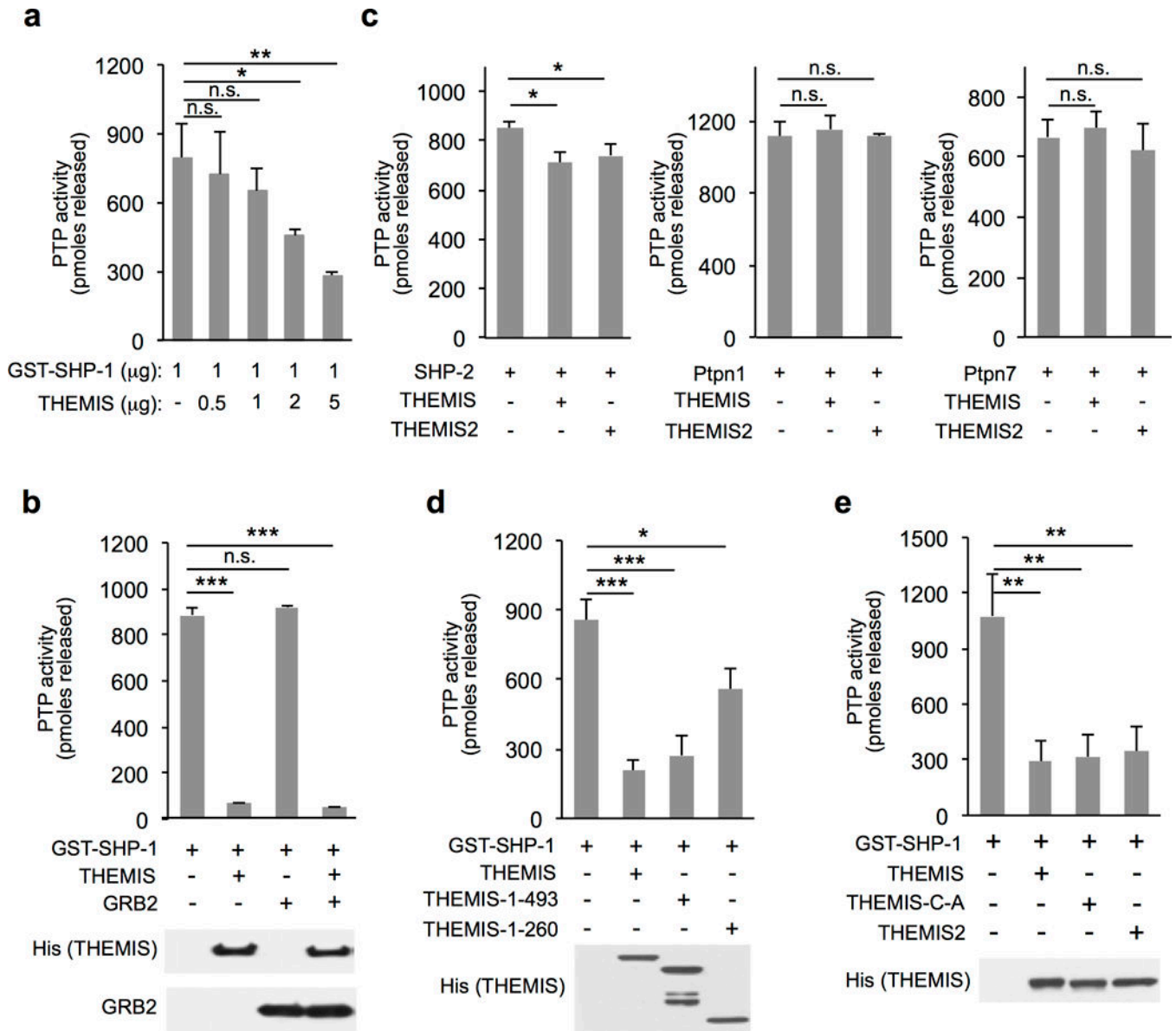
Figure 2. THEMIS directly inhibits SHP-1 tyrosine phosphatase activity
a, *in vitro* GST-SHP-1 protein tyrosine phosphatase (PTP) assay performed in the presence of increasing amounts of THEMIS. Amount of each protein added is shown below the bar graph. n=3. **b,** *in vitro* GST-SHP-1 PTP assay performed in the presence of no added protein, THEMIS or GRB2. Blots show input of THEMIS or GRB2. n=3. **c,** *in vitro* PTP assay of SHP-2, PTPN1 or PTPN7 performed in the presence or absence of THEMIS or THEMIS2. n=3 each. **d,e,** *in vitro* PTP assays of GST-SHP-1 performed in the presence or absence of THEMIS, THEMIS1-493 encoding CABIT1 + CABIT2, THEMIS1-260 encoding CABIT1, THEMIS-C-A containing C to A mutations of the CABIT1 and CABIT2 core cysteines, or THEMIS2. n=5 for GST-SHP-1 alone or GST-SHP-1 with THEMIS, n=3 for GST-SHP-1 with each THEMIS variant. In **b-e**, all non-PTP proteins were added at a 5:1 molar ratio relative to the PTP. In **b,d,e**, blots show input of THEMIS or THEMIS variant

proteins. Bar graphs show means + SD, t-test. *P<.05, **P<.01, ***P<.005. n.s., not significant (P>.05).
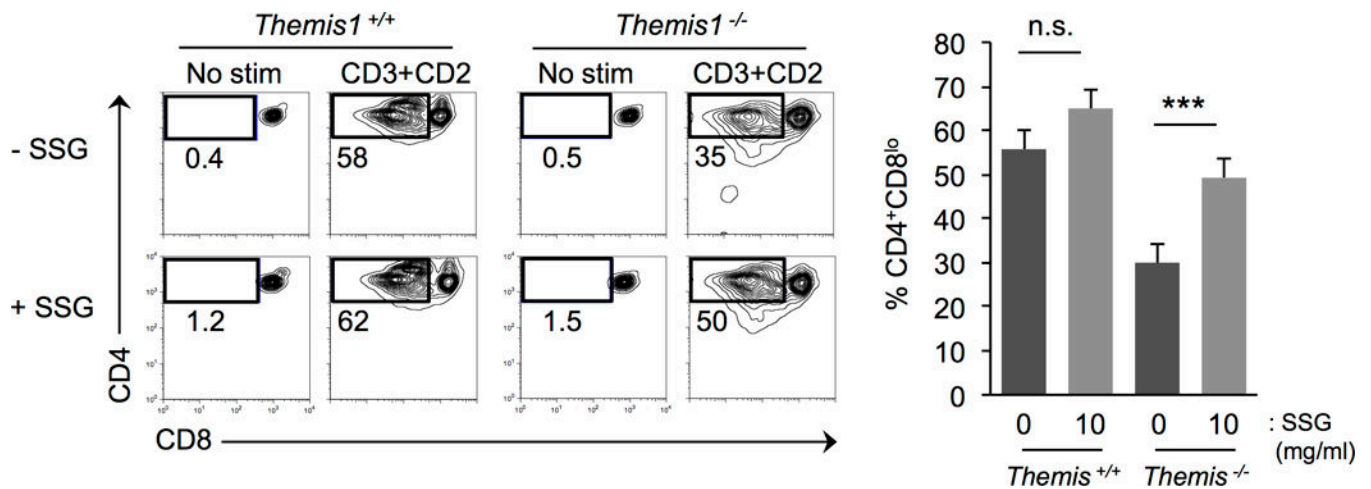
Figure 3. Inhibition of SHP-1 PTP activity rescues *in vitro* maturation of *Themis* $^{-/-}$ thymocytes
Differentiation of DP thymocytes from Themis $^{-/-}$ or *Themis* $^{+/+}$ to the CD4$^+$CD8$^-$ stage in a two step (stimulation-rest) *in vitro* assay with or without the SHP-1 inhibitor Sodium stibogluconate (SSG). Left, two parameter plots show CD4 versus CD8 staining profiles of thymocytes at the completion of the differentiation assay. Cell recovery and % apoptotic (Annexin V$^+$) cells were not significantly different in similarly treated *Themis* $^{-/-}$ or *Themis* $^{+/+}$ samples. Right, Summary of results. n=4 for each genotype (t-test 2-tailed type-2, error bars show SD). ***P<.005. n.s., not significant (P>.05).
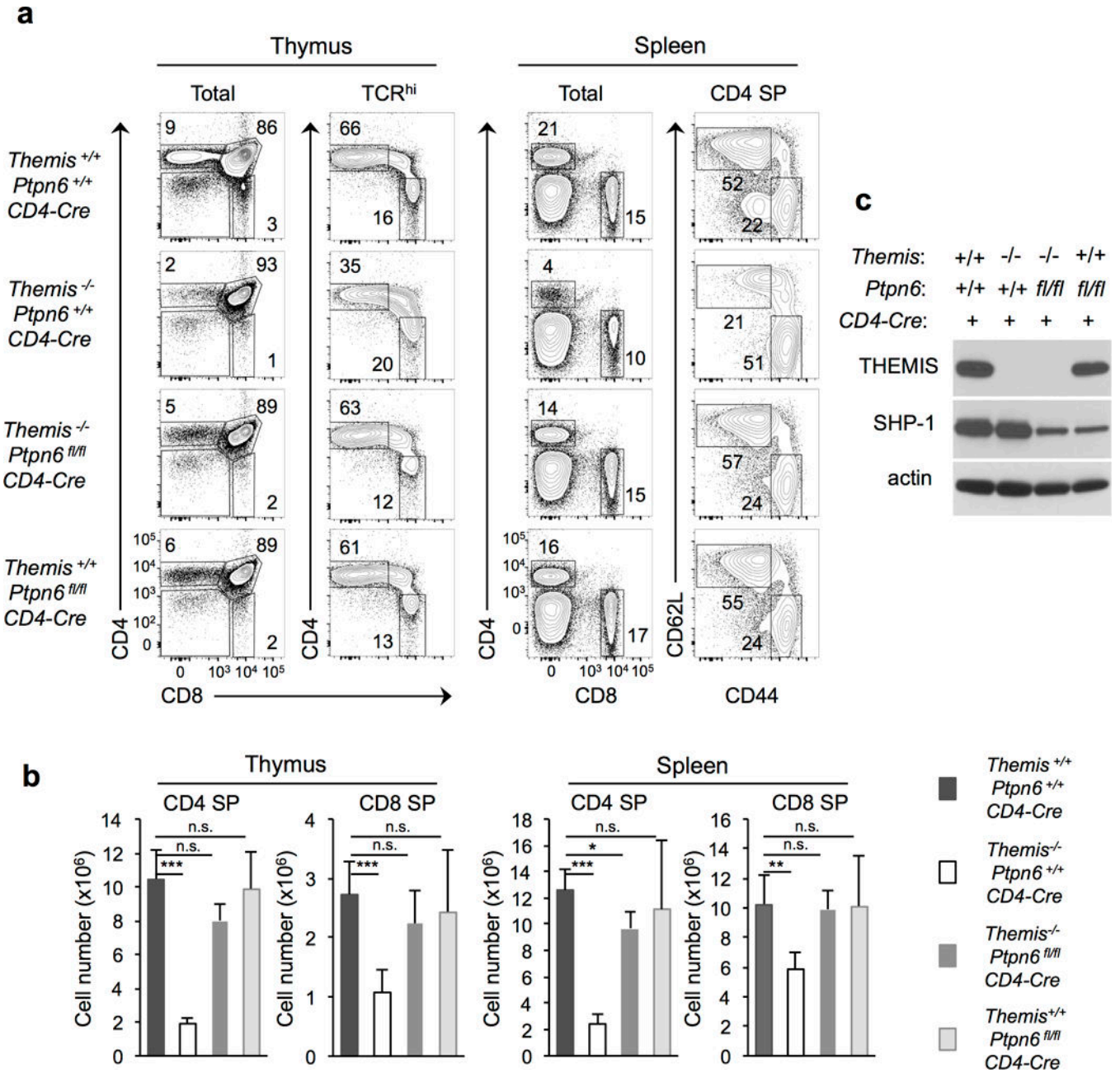
Figure 4. Reduction of SHP-1 alleviates the developmental block in *Themis*$^{-/-}$ thymocytes

a, Flow cytometry analysis of thymocytes from mice of the indicated genotype. Thymus: Two parameter plots show CD4 versus CD8 staining on total thymocytes (left panels) or gated TCR^{hi} thymocytes (right panels). Spleen: Two parameter plots show CD4 versus CD8 staining on total splenocytes (left panels) or CD44 versus CD62L staining on gated CD4 SP T cells (right panels). **b**, Enumeration of CD4 SP and CD8 SP cells in the Thymus and Spleen from mice of the genotype indicated in **a**. Bar graphs show means + SD, t-test; n=4 mice of each genotype. *$P<$.05, **$P<$.01, ***$P<$.005. n.s., not significant ($P>$.05). Key for bar graphs is shown on the right. **c**, Expression of THEMIS and SHP-1 in thymocytes from the experiment shown in **a** (one representative of four).
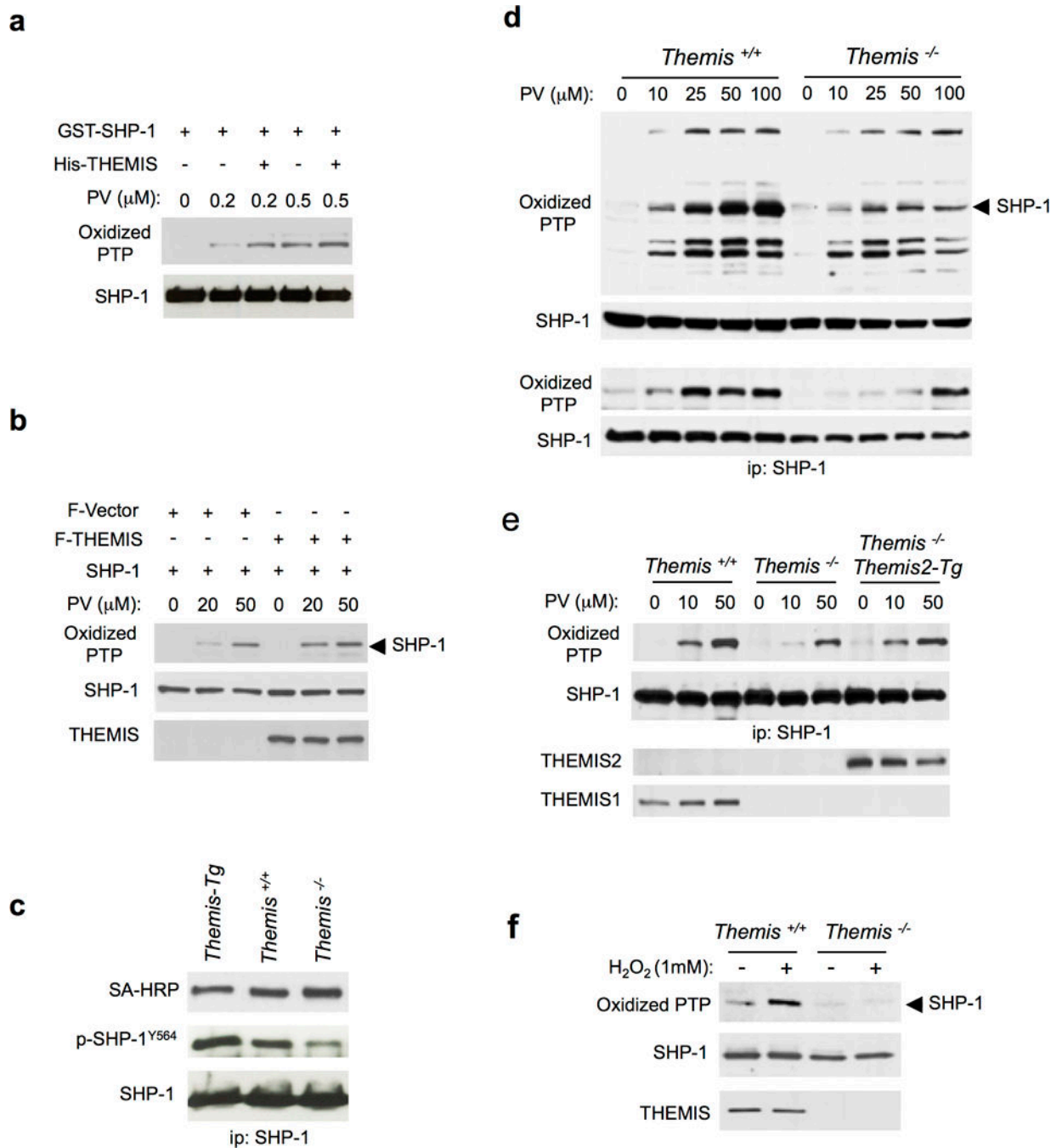
a

GST-SHP-1   +   +   +   +   +
His-THEMIS   -   -   +   -   +
PV (μM):   0   0.2   0.2   0.5   0.5

Oxidized PTP

SHP-1

d

      Themis [+/+]   *Themis* [-/-]
PV (μM):   0   10   25   50   100   0   10   25   50   100

Oxidized PTP   ◄ SHP-1

SHP-1

Oxidized PTP

SHP-1

ip: SHP-1

b

F-Vector   +   +   +   -   -   -
F-THEMIS   -   -   -   +   +   +
SHP-1   +   +   +   +   +   +
PV (μM):   0   20   50   0   20   50

Oxidized PTP   ◄ SHP-1

SHP-1

THEMIS

e

                         Themis [-/-]
   Themis [+/+]   *Themis* [-/-]   *Themis2-Tg*
PV (μM):   0   10   50   0   10   50   0   10   50

Oxidized PTP

SHP-1

ip: SHP-1

THEMIS2

THEMIS1

c

      Themis-Tg   *Themis* [+/+]   *Themis* [-/-]

SA-HRP

p-SHP-1[Y564]

SHP-1

ip: SHP-1

f

      Themis [+/+]   *Themis* [-/-]
H₂O₂ (1mM):   -   +   -   +

Oxidized PTP   ◄ SHP-1

SHP-1

THEMIS

Figure 5. THEMIS promotes or stabilizes oxidation of SHP-1

a, *in vitro* active site oxidation of GST-SHP-1 after treatment with pervanadate (PV) in the presence or absence of THEMIS. Shown are blots of proteins after SDS-PAGE. One representative of two experiments. **b,** Active site oxidation of SHP-1 by PV in transfected HEK-293 cells. F, Flag epitope tag. SHP-1 band is identified by arrow. One representative of two experiments. **c,** Assay for catalytically active (reduced) SHP-1 in total thymocytes from the indicated mice. Active SHP-1 was detected by labeling with IAP-bio and blotting with SA-HRP (Streptavidin-Horseradish peroxidase). Results shown are representative of 6

experiments. **d**, Active site oxidation of SHP-1 in thymocytes treated with PV. Upper panel, blots of cell lysates. SHP-1 band is identified by arrow. Lower panel, blots of immunoprecipitated SHP-1. One representative of two experiments. **e**, THEMIS2 increases the sensitivity of SHP-1 to oxidation by pervanadate. Thymocytes were treated and analyzed as described in **d**. Upper panel, blots of immunoprecipitated SHP-1. Lower panels, blots of cell lysates. Results are representative of 3 experiments. **f**, Active site oxidation of SHP-1 in thymocytes treated with H_2O_2. SHP-1 band is identified by arrow. Results shown are representative of 3 experiments.
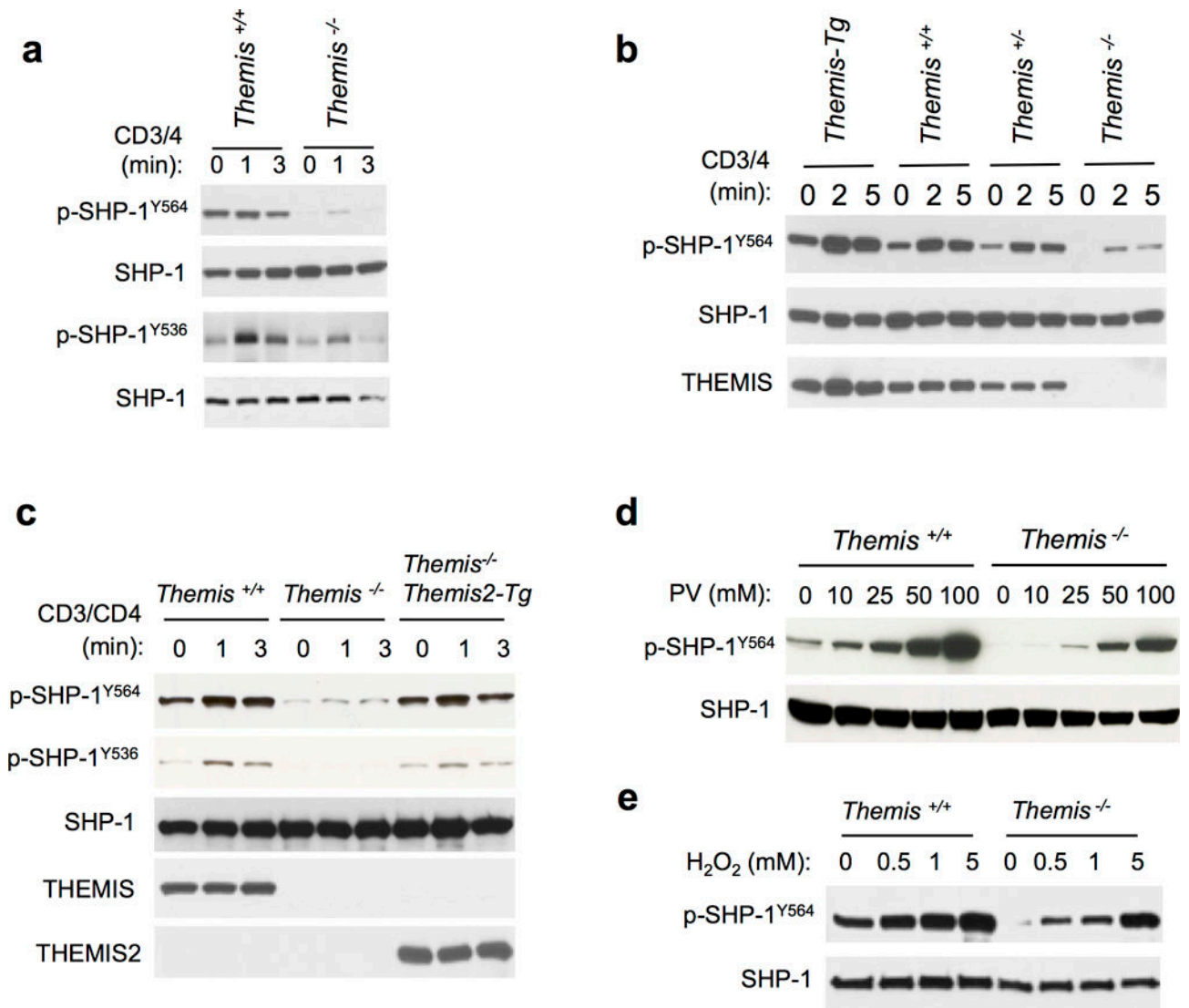
Figure 6. Reduced tyrosine phosphorylation of SHP-1 in *Themis*^−/− thymocytes is caused by increased SHP-1 PTP activity

a, Analysis of tyrosine phosphorylated SHP-1 (p-SHP-1) at Y536 and Y564 in un-stimulated and stimulated total thymocytes from *Themis*^+/+ and *Themis*^−/− mice by immunoblotting. Results are representative of 3 experiments. **b.**, Analysis of p-SHP-1 in un-stimulated and stimulated total thymocytes from *Themis*-transgenic (Tg); *Themis*^+/+, *Themis*^+/−, and *Themis*^−/− mice by immunoblotting. Results are representative of 4 experiments. **c**, Analysis of p-SHP-1 in total thymocytes from the indicated mice before and after CD3+4 stimulation. Results are representative of 3 experiments. **d,e**, Effect of the PTP inhibitors pervanadate(PV) (**d**) or H_2O_2 (**e**) on p-SHP-1 levels in total thymocytes from *Themis*^+/+ and *Themis*^−/− mice. Blots shown are representative of 6 experiments.
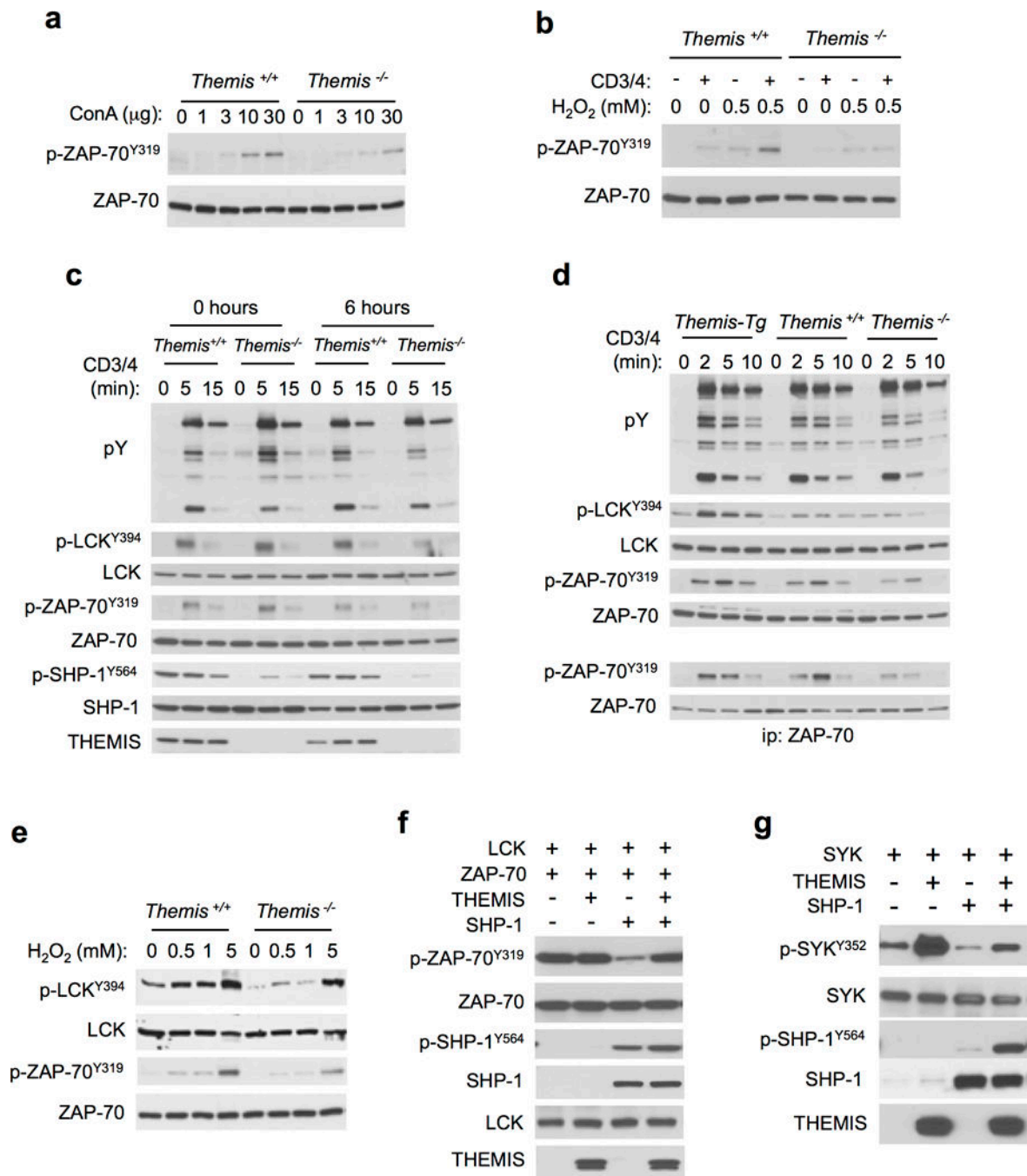
Figure 7. Attenuated TCR signaling responses in *Themis*^{−/−} thymocytes in the presence of ROS

a,b, p-ZAP-70 induction in *Themis*^{+/+} and *Themis*^{−/−} total thymocytes stimulated with (**a**) ConA or (**b**) anti-CD3/4 plus H_2O_2. Results shown are representative of 3 experiments. **c**, Evaluation of signaling responses to anti-CD3/4 of total thymocytes from *Themis*^{+/+} or *Themis*^{−/−} mice after 0 h or 6h culture at 37°C in serum free medium. Results are representative of 4 experiments. **d**, Evaluation of signaling responses of total thymocytes from *Themis-Tg*, *Themis*^{+/+} or *Themis*^{−/−} mice after 6h culture at 37°C in serum free medium. Results are representative of 3 experiments. **e**, Induction of p-LCK and p-ZAP-70

in total thymocytes from *Themis*$^{+/+}$ or *Themis*$^{-/-}$ by H_2O_2. Results are representative of 3 experiments. **f**, Assay of SHP-1-mediated de-phosphorylation of ZAP-70 in transfected HEK-293 cells in the presence or absence of THEMIS. Plasmids included in each transfection are noted above each lane. Plasmid encoding LCK was included in all tranfections to induce phosphorylation of ZAP-70. Results are representative of 2 experiments. **g**, Assay of SHP-1-mediated de-phosphorylation of SYK in transfected HEK-293 cells in the presence or absence of THEMIS. Plasmids included in each transfection are noted above each lane. Results are representative of 2 experiments.