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

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THEMIS is a T cell–specific protein with high expression 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 module of unknown function (CABIT). Here we found that THEMIS directly regulated the catalytic activity of the tyrosine phosphatase SHP-1. This action was mediated by the CABIT modules, which bound to the phosphatase domain of SHP-1 and promoted or stabilized oxidation of SHP-1’s catalytic cysteine residue, which inhibited the tyrosine-phosphatase activity of SHP-1. Deletion 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.

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 cell lineage. Thymocytes progress through multiple well-defined maturational steps that for simplicity are grouped into three main stages defined by expression of the co-receptors CD4 and CD8: CD4+CD8− (double negative (DN)), CD4+CD8+ (double positive (DP)), and CD4−or CD8+ (single positive: CD4SP or CD8SP, respectively). The transition of thymocytes through these stages of maturation is dependent upon signals transmitted by various 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 that is based on the affinity of their expressed TCR for self-peptide ligands bound to major histocompatibility complex (self-pMHC) that tests TCR functionality and enforces self-tolerance1. 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 death, whereas thymocytes that express TCRs that bind with low affinity to self-pMHC are ‘positively selected’ and progress to the CD4SP or CD8SP stage1. The affinity of the TCR for self-pMHC 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 fate4.

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 (encoded by Ptpn6), and specialized adaptors such as LAT and SLP-76 (refs. 5,6). THEMIS, a T cell–specific protein, has an important role in thymocyte selection. In the absence of THEMIS, thymocyte development is partially blocked at the DP-to-SP transition stage, which results in a substantial reduction in mature CD4SP thymocytes and, to lesser extent, CD8SP thymocytes and peripheral T cells7–11.

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 CABIT (cysteine-containing all beta in THEMIS) globular module with a median length of 261 amino acids that contains a conserved core motif (ϕXCX7−9ϕXPϕϕGϕϕ, where ‘ϕ’ is any hydrophobic residue, ‘X’ is any amino acid, and the subscripted number indicates the number of residues)9. All members of the mammalian THEMIS family, including THEMIS, THEMIS2 (which is restricted to B cells and myeloid cells) and the more distantly related THEMIS3 (which is expressed in the large and small intestine12) contain two tandem CABIT modules and a C-terminal proline-rich sequence but lack a known catalytic domain9. THEMIS binds directly to the cytosolic adaptor GRB2, and this interaction requires the proline-rich sequence of THEMIS12,13. Mass-spectrometry screens of proteins co-immunoprecipitated with THEMIS have identified SHP-1 as a putative THEMIS-interacting protein14,15, and it has been suggested that THEMIS functions by regulating the activity of SHP-1 or its recruitment to LAT16,17. Nevertheless, a specific role for THEMIS in T cell development has not been clearly defined, and it remains unclear if

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its effect on TCR signaling is mainly activating or inhibitory.\textsuperscript{14-16} A particular challenge has been to identify a function for the CABIT modules that constitute most of the THEMIS protein. The presence of highly conserved core sequences and their requirement for THEMIS activity in vivo\textsuperscript{18} 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.\textsuperscript{8}

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

RESULTS

Binding of THEMIS to the PTP domain of SHP-1

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 of HEK-293 human embryonic kidney cells co-transfected with plasmids encoding SHP-1 and THEMIS lacking the GRB2-binding proline-rich sequence (THEMIS-1–493), SHP-1 immunoprecipitated together 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 Src-homology-2 (SH2) PTP that is closely related to SHP-1) bound to THEMIS when co-expressed in HEK-293 cells, but two other class I PTPs (PTPN1 and PTPN7) that are expressed in thymocytes did not bind to THEMIS (Supplementary Fig. 1a,b). To locate the sequences within THEMIS that mediated its binding to SHP-1, we next performed co-immunoprecipitation experiments in HEK-293 cells transfected with plasmids encoding SHP-1 and various truncations of THEMIS. SHP-1 immunoprecipitated together with a THEMIS protein that contained only the CABIT1 and CABIT2 modules (THEMIS-1–493) and, to a lesser extent, with truncated THEMIS proteins containing only the CABIT1 module (THEMIS-1-260) or CABIT2 module (THEMIS-260–493) (Fig. 1b). In similar co-transfection experiments, a protein containing only the PTP domain of SHP-1 immunoprecipitated together with THEMIS-1–493 and with THEMIS-1–260 (Fig. 1d). Also, purified SHP-1 lacking both SH2 domains but containing the PTP domain (GST–ΔSH2–SHP-1) bound to THEMIS-1–493 in vitro (Fig. 1e and Supplementary Fig. 1c,d).

**Figure 1** THEMIS binds directly to SHP-1. (a) In vitro GST-precipitation assay of GST–SHP-1, assessing the binding of THEMIS to SHP-1 in the presence (+) or absence (−) of GRB2. (b) Co-immunoprecipitation analysis (below) of the binding of THEMIS CABIT modules to SHP-1 in HEK-293 cells co-transfected with Flag (F) parent plasmid or plasmid encoding various Flag-tagged (F-) THEMIS constructs (top) plus plasmids encoding SHP-1 and Myc-tagged GRB2 (above lanes), assessed by immunoblot analysis of cell lysates (bottom two blots) or proteins immunoprecipitated with anti-Flag (IP: Flag; top three blots). PRS, proline-rich sequence. (c) Co-immunoprecipitation analysis of THEMIS and SHP-1 in lysates of total thymocytes from Themis<sup>+/−</sup>, Themis<sup>−/−</sup> and GRB2-deficient (Grb2<sup>fl/fl</sup>–Lcr-Cre) mice (above lanes). (d) Co-immunoprecipitation analysis (below) of the binding of THEMIS CABIT modules to the PTP domain of SHP-1 in HEK-293 cells co-transfected to express various Flag-tagged THEMIS constructs (as in b) plus plasmid encoding SHP-1 or its PTP domain (top) (above lanes), assessed by immunoblot analysis of lysates or immunoprecipitated proteins as in b. (e) In vitro GST-precipitation (ppt) assay of GST–SHP-1, assessing the binding of histidine tagged THEMIS CABIT modules (His-1–493) to the PTP domain of SHP-1 (ΔSH2–SHP-1 construct, d). Data are representative of three experiments.
Together these results demonstrated that the CABIT modules of THEMIS interacted directly with the PTP domain of SHP-1.

Inhibition of SHP-1’s PTP activity by the THEMIS CABIT modules

To determine if THEMIS directly regulates the PTP activity of SHP-1, we used an in vitro assay to detect the release of phosphate from a tyrosine-phosphorylated peptide. The PTP activity of SHP-1 was diminished in the presence of THEMIS, and this correlated with the concentration of THEMIS (Fig. 2a). The PTP activity of SHP-1 was not inhibited by GRB2, which also binds to SHP-1, which demonstrated that the inhibitory effect was specific to THEMIS; however, the 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 diminished slightly by THEMIS, whereas THEMIS did not inhibit the PTP activity of PTPN1 or PTPN7 (Fig. 2c). THEMIS-1–493 was nearly as effective as full-length THEMIS at inhibiting the PTP activity of SHP-1 (Fig. 2d), which indicated that the CABIT modules were responsible for this inhibitory function of THEMIS. THEMIS-1–260 also inhibited the PTP activity of SHP-1, although not as effectively as THEMIS-1–493, which contains both CABIT modules (Fig. 2d); this indicated that a single CABIT module contained the sequences necessary for regulating the PTP activity of SHP-1 but that both CABIT modules were required for full inhibition. THEMIS2, which ‘rescues’ the developmental block in Themis−/− thymocytes when transgenically expressed in thymocytes, and therefore could substitute for THEMIS in vivo, inhibited the PTP activity of SHP-1 (Fig. 2e) and, to a lesser extent, that of SHP-2, but not that of PTPN1 or PTPN7, in in vitro tyrosine-phosphatase assays (Fig. 2e).

All mammalian CABIT modules contain a conserved cysteine residue (underlined below) within the core motif of φXGX2_3φXGX2_3φXGX2_3φXGX2_3φXGX2_3φXG of the cysteine residue in the regulatory activity of THEMIS, we introduced cysteine-to-alanine point substitutions in both the CABIT1 module (Cys153) and CABIT2 module (Cys413) of THEMIS (THEMIS-C-A). THEMIS-C-A immunoprecipitated together with SHP-1 in lysates of HEK-293 cells transfected to express THEMIS-C-A and SHP-1, in in vitro tyrosine-phosphatase assay (Fig. 2e), which indicated that the cysteine residues in the CABIT domains were not essential for regulating the PTP activity of SHP-1. This was consistent with published observations that retrovirally encoded THEMIS-C-A is able to ‘rescue’ the DP-to-SP developmental block in Themis−/− thymocytes.
results demonstrated that the THEMIS CANT modules directly inhibited the PTP activity of SHP-1 and that the conserved core cysteine was not required for this function.

**Deletion of *Ptpn6* restores T cell development in *Themis*−/− mice**

We next determined if inhibition of the PTP activity of SHP-1 would reverse the developmental block in *Themis*−/− thymocytes in an *in vitro* differentiation assay. Overnight culture of immature (TCRβ) DP thymocytes with plate-bound antibody to the TCR invariant chain CD3e (anti-CD3e) plus antibody to the adhesion molecule CD2 (anti-CD2), followed by 24 h of rest without stimulation, induces their progression to the CD4+CD8lo stage, which replicates the initial stages of positive selection *in vivo*. In contrast to wild-type DP thymocytes, *Themis*−/− DP thymocytes exhibited an impaired ability to transition to the CD4+CD8lo stage (Fig. 3). The block in the progression of *Themis*−/− DP thymocytes to the CD4+CD8lo stage was significantly alleviated when the *in vitro* assay was performed in the presence of sodium stibogluconate, a selective inhibitor of SHP-1 (ref. 22) (Fig. 3); this indicated that the enhanced PTP activity of SHP-1 in *Themis*−/− thymocytes contributed to the developmental defect.

To determine if the developmental block in *Themis*−/− thymocytes could be ‘rescued’ by a reduction in the expression of SHP-1, we generated *Themis*−/− *Ptpn6fl/fl*CD4-Cre mice, in which T cell–lineage-specific deletion of *loxP*-flanked alleles encoding SHP-1 (*Ptpn6fl/fl*), via Cre recombinase expressed via the T cell–specific Cd4 promoter, occurs mainly in DP thymocytes. Thymocytes from *Themis*−/− *Ptpn6+/−*CD4-Cre control mice exhibited normal maturation up to the DP stage but a marked reduction in the number of CD4SP thymocytes and, to lesser extent, CD8SP thymocytes and peripheral T cells (Fig. 4a,b), similar to the developmental phenotype of *Themis*−/− mice. The expression of SHP-1 in total thymocytes from *Themis*−/− *Ptpn6*+/−*CD4-Cre mice was reduced to approximately 25% that observed in total thymocytes from *Themis*−/− *Ptpn6+/−*CD4-Cre mice, as assessed by densitometry (Fig. 4c). This reduction in the expression of SHP-1 alleviated the developmental block, as evinced by the significantly greater frequency and number of mature *TCRβhi* CD4SP and CD8SP thymocytes and peripheral T cells in *Themis*−/− *Ptpn6+/*CD4Cre mice than in *Themis*−/− *Ptpn6+/−*CD4-Cre mice (Fig. 4a,b). In contrast to *Themis*−/− *Ptpn6+/−*CD4-Cre mice, which had a large frequency of peripheral T cells with a CD62Llo-CD44hi memory T cell phenotype as a result of lymphopenia-induced expansion, the frequency of CD62Llo-CD44hi T cells in *Themis*−/− *Ptpn6+/−*CD4-Cre mice was similar to that in *Themis*+/+ *Ptpn6+/−*CD4-Cre mice (Fig. 4a). Deletion of *Ptpn6* at an earlier stage of development (predominantly at the DN stage) through the use of an *LCK*-Cre transgene resulted in a nearly complete absence of SHP-1 protein in total thymocytes and also substantially alleviated the developmental block caused by the deletion of *Themis* (Supplementary Fig. 2). Together these results indicated that the developmental defect in *Themis*−/− thymocytes was due to the enhanced PTP activity of SHP-1.

**Regulation of active-site oxidation of SHP-1 by THEMIS**

All classical PTPs, including SHP-1 and SHP-2, contain a conserved active-site cysteine residue (with an unusually low acidic dissociation constant) that catalyzes the removal of phosphate from phosphorylated tyrosines. 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 inactivate the PTP. To determine if the 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-OH) form, to cell-free suspensions of GST–SHP-1 in the presence or absence of THEMIS. Oxidized SHP-1 was detected by immunoblot analysis with a monoclonal antibody specific for sulfonylated PTP active-site cysteines. Oxidation of SHP-1 by pervanadate was enhanced in the presence of THEMIS, and this effect was most evident at concentrations of pervanadate that resulted in sub-maximal oxidation of SHP-1 (Fig. 5a and Supplementary Fig. 3a). THEMIS also increased the susceptibility of SHP-1 to oxidation by pervanadate in HEK-293 cells co-transfected to express THEMIS and SHP-1 (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 the addition of iodoacetyl–polyethylene glycol–biotin, which immediately and irreversibly binds to reduced, de-protonated (−S) cysteine thiols. The abundance of catalytically active SHP-1 was slightly but consistently greater in *Themis*−/− total thymocytes and was lower in total thymocytes with transgenic expression of THEMIS14 than in *Themis*+/+ (control) total thymocytes (Fig. 5c). Lysis of thymocytes in the absence of PTP inhibitors resulted 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 immunoprecipitated from thymocyte lysates by the tyrosine-phosphatase assay. However, SHP-1 was much less susceptible to oxidation by pervanadate in *Themis*−/− total thymocytes than in *Themis*+/+ total thymocytes (Fig. 5d and Supplementary Fig. 3e). The susceptibility of SHP-1 to oxidation by pervanadate was restored in *Themis*−/− thymocytes with transgenic expression of THEMIS2 (ref. 13) (Fig. 5e), which demonstrated...
a function shared by THEMIS and THEMIS2. The activation of T cells and B cells results in the production of ROS, predominantly H$_2$O$_2$, and this effect has been shown to positively regulate both TCR and BCR signaling and effector responses through oxidative inhibition of SHP-1 (refs. 26–28). In the presence of THEMIS, active-site oxidation of SHP-1 by H$_2$O$_2$, assessed by immunoblot analysis with antibody to sulfonylated PTP active sites, was markedly increased (Fig. 5f), which indicated that THEMIS regulated the redox state of SHP-1 in response to physiological ROS. Together these results demonstrated that THEMIS inhibited the PTP activity of SHP-1 by promoting or stabilizing ROS-mediated oxidation of the active-site cysteine residue of SHP-1.

**p-SHP-1 does not correspond with PTP activity**

We found that phosphorylation of SHP-1, which occurs at two C-terminal tyrosine residues (Tyr336 and Tyr564), was lower in Themis$^{-/-}$ total thymocytes than in Themis$^{+/+}$ total thymocytes (Fig. 6a,b), in confirmation of published results. The amount of tyrosine-phosphorylated SHP-1 (p-SHP-1) in total thymocytes correlated with the expression of THEMIS protein, and the amount of p-SHP-1 was restored in Themis$^{-/-}$ thymocytes by expression of a transgene encoding THEMIS2 (ref. 13) (Fig. 6a–c). The lower abundance of p-SHP-1 in Themis$^{-/-}$ thymocytes has been interpreted as evidence of diminished catalytic activity of SHP-1 (ref. 16); however, phosphorylation of SHP-1 is not required for its PTP catalytic activity, and its physiological relevance has not been established. The amount of catalytically active SHP-1 in unstimulated Themis$^{-/-}$ total thymocytes was greater than, not less than, that in Themis$^{+/+}$ total thymocytes (Fig. 5c, d). Because SHP-1 is a target of SHP-1’s phosphatase activity, we reasoned that the diminished amount of p-SHP-1 in Themis$^{-/-}$ thymocytes was secondary to increased auto- or trans-dephosphorylation by SHP-1. Indeed, treatment of both Themis$^{+/+}$ total thymocytes and Themis$^{-/-}$ total thymocytes with pervanadate or H$_2$O$_2$, which inhibit SHP-1 activity, led to an increase in p-SHP-1 relative to its abundance in their untreated counterparts (Fig. 6d, e). Together these results demonstrated that SHP-1’s phosphorylation status could not be used to predict its PTP catalytic activity and suggested that the lower abundance of p-SHP-1 in Themis$^{-/-}$ thymocytes was secondary to enhanced auto- or trans-dephosphorylation by SHP-1.

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

In contrast to mature T cells, in which engagement of the TCR induces the production of ROS, stimulation of thymocytes via the TCR failed to elicit ROS (Supplementary Fig. 4a). However, ROS are induced in thymocytes by stimulation with the lectin concanavalin A, which engages multiple cell-surface molecules in addition to the TCR. Tyrosine-phosphorylation of the PTK ZAP-70 (p-ZAP-70), a known target of SHP-1 (refs. 34–35), at Tyr319 was lower in Themis$^{-/-}$ total thymocytes than in Themis$^{+/+}$ total thymocytes following stimulation with concanavalin A (Fig. 7a), whereas there was no difference between Themis$^{-/-}$ total thymocytes and Themis$^{+/+}$ total thymocytes in their abundance of p-ZAP-70 following stimulation with anti-CD3 plus anti-CD4 (Fig. 7b,c). However, when H$_2$O$_2$ was added at the time of stimulation with anti-CD3 plus anti-CD4, the tyrosine-phosphorylation of ZAP-70 induced by this was lower in Themis$^{-/-}$ total thymocytes than in 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. Freshly harvested Themis$^{-/-}$ total thymocytes exhibited no clear defects in proximal TCR signaling responses relative to those of Themis$^{+/+}$ total thymocytes (Fig. 7c). However, after in vitro culture for 6 h, the tyrosine phosphorylation of ZAP-70, as well as the tyrosine-phosphorylation of LCK (phosphorylated at Tyr394), another putative target of SHP-1 (ref. 37), was lower in Themis$^{-/-}$ total thymocytes than in Themis$^{+/+}$ total thymocytes in response to either stimulation with anti-CD3 plus anti-CD4 (Fig. 7c, d) or stimulation with peptide presented by

![Figure 4](Image)

**Figure 4** Deletion of Ptpn6 alleviates the developmental block in Themis$^{-/-}$ thymocytes. (a) Flow cytometry of cells from the thymus (left) or spleen (right) of Themis$^{+/+}$ Ptpn6$^{+/+}$ Cd4-Cre mice, Themis$^{-/-}$ Ptpn6$^{-/-}$ Cd4-Cre mice, Themis$^{-/-}$ Ptpn6$^{+/+}$ Cd4-Cre mice or Themis$^{+/+}$ Ptpn6$^{+/+}$ Cd4-Cre mice (left margin), assessing the staining of CD8 versus CD4 on total thymocytes (left column) or gated TCR$^{hi}$ thymocytes (right column) (thymus), or the staining CD4 versus CD8 on total splenocytes (left column) or staining of CD62L versus CD44 on gated CD4SP T cells (right column) (spleen). Numbers adjacent to outlined areas indicate percent cells in each gated area. (b) Quantification of CD4SP cells and CD8SP cells in the thymus and spleen of mice of the genotypes in a (key). (c) Immunoblot analysis of THEMIS, SHP-1 and actin (loading control) in the thymocytes in a (genotypes, above lanes). *P < 0.05, **P < 0.01 and ***P < 0.005 (t-test). Data are from one experiment representative of four experiments with n = 1 mouse per genotype in each a, c or are from four experiments with n = 1 mouse per genotype in each b; mean ± s.d.)
antigen-presenting cells (Supplementary Fig. 4b). Themis+/– total thymocytes also exhibited less induction of tyrosine phosphorylation of LCK and ZAP-70 in response to treatment with H₂O₂ alone than that of Themis+/+ total thymocytes treated in an identical way (Fig. 7c). De-phosphorylation of ZAP-70 or of the related B cell PTK SYK by SHP-1 was inhibited by THEMIS in HEK-293 cells transfected with plasmids encoding ZAP-70 or SYK plus plasmid encoding SYK and cultured under conditions in which ROS are constitutively produced after treatment with various concentrations (above lanes) of pervanadate (PV) in the presence or absence of histidine-tagged (His-) THEMIS (above lanes). (b) Immunoblot analysis of the active-site oxidation of SHP-1 in HEK-293 cells transfected to express a Flag epitope vector (F) or Flag-tagged (F-) THEMIS (above lanes) and treated with various concentrations of peroxidase; arrowhead (right margin) indicates SHP-1 (thymocytes, The diminished tyrosine-phosphorylation of SHP-1 in Themis−/− thymocytes is caused by increased PTP activity of SHP-1. (a) Immunoblot analysis of SHP-1 phosphorylated at Tyr536 (p-SHP-1(Y536)) or Tyr564 (p-SHP-1(Y564)) and total SHP-1 (left margin) in Themis+/+ and Themis−/− total thymocytes left unstimulated (0) or stimulated for 1 or 3 min (above lanes) with anti-CD3 plus anti-CD4 (CD3+CD4). (b) Immunoblot analysis of SHP-1 phosphorylated at Tyr564 and total SHP-1, as well as total THEMIS (left margin), in total thymocytes obtained from mice with transgenic expression of THEMIS, and Themis−/− or Themis+/− mice (above blots). (c) Immunoblot analysis of tyrosine-phosphorylated and total SHP-1 (as in a, left margin) in total thymocytes obtained from Themis−/− and Themis−/− mice and Themis−/− mice with transgenic expression of THEMIS2 (above blots) and stimulated as in a (above lanes). (d,e) Immunoblot analysis of tyrosine-phosphorylated and total SHP-1 (as in b, left margin) in total thymocytes obtained from Themis+/+ and Themis−/− mice and treated with various concentrations (above lanes) of peroxidate (d) or H₂O₂ (e). Data are representative of three experiments (a,c), four experiments (b) or six experiments (d,e).}

**DISCUSSION**

Here we have shown that a critical function of THEMIS during T cell development is to negatively regulate the activity of the PTP SHP-1 in DP thymocytes and thereby enhance the TCR signaling response to low-affinity self-pMHC and enable positive selection. THEMIS promoted or stabilized oxidation of the catalytic cysteine of SHP-1, which inhibited its PTP activity, and this regulatory activity was conferred by the CABIT modules that bound directly to the PTP domain of SHP-1.

While it remains to be determined how the CABIT modules regulate the oxidation of SHP-1, several plausible mechanisms can be suggested on the basis of their inferred structure together with what is already known about the structure and redox regulation of SHP-1. CABIT modules, which are composed of multiple SH3-like β-barrel domains, probably form an extensive protein-binding globular interface that specifically recognizes the PTP domain of SHP-1. The catalytic cysteine residue of all classical PTPs is housed inside a pocket with an aperture that allows the entry of only the phosphate moiety on tyrosine residues. Thus, binding of the CABIT modules might prevent access of the oxidized catalytic cysteine of SHP-1 to reducing agents such as glutathione in the bulk solvent or to cytosolic...
redox-regulatory proteins\(^\text{39}\). Alternatively, the CABIT modules might stabilize the PTP domain of SHP-1 in an unfolded state, which would expose the catalytic cysteine to oxidation by ROS or prevent reactivation of the oxidized catalytic cysteine by inhibiting intra-molecular relay of sulfenic acid to the two regulatory cysteine residues (Cys329 and Cys363) in SHP-1 (refs. \(^\text{39}\)). We note that our \textit{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 probably also blocks access of the catalytic cysteine to phosphorylated-tyrosine ligands, a mechanism of inhibition that is not mutually exclusive with redox regulation.

Although the CABIT modules of THEMIS bound directly to SHP-1, experimental data suggest that the interaction of THEMIS with GRB2 is important for its \textit{in vivo} function\(^\text{12,21,31}\). Co-binding of SHP-1 and THEMIS to GRB2 brings these proteins into close proximity and might facilitate and stabilize their direct interaction. In addition, following engagement of the TCR, GRB2, via its SH2 domain, recruits SHP-1 to tyrosine-phosphorylated ligands at the cell membrane, including LAT and CD28 (ref. \(^\text{42}\)). Thus, the binding to GRB2 ensures that THEMIS is positioned to affect the activity of the cellular portion of SHP-1 that is presumably the most relevant to TCR signaling. Finally, GRB2 might also be needed to position SHP-1 and THEMIS near sites of ROS production by NADPH oxidases at the cell membrane.

It has been proposed that THEMIS enhances the activity of SHP-1, either directly or by assisting in its recruitment to LAT, and thereby acts to dampen TCR signaling in DP thymocytes\(^\text{16}\). According to that model, the PTP activity of SHP-1 is diminished in \textit{Themis} \(^{-/}\) DP thymocytes, and engagement of the TCR by low-affinity ligands that normally promote positive selection results in the transduction of enhanced signaling responses that trigger negative selection\(^\text{16}\). Our results do not support that model. If the thymocyte-maturational defect of \textit{Themis} \(^{-/}\) mice was caused by diminished SHP-1 activity, deletion of \textit{Ptpn6} or inhibition of the PTP activity of SHP-1 should not have ‘rescued’, and might possibly have exacerbated, the block in T cell development in \textit{Themis} \(^{-/}\) mice. Instead, inhibition of the PTP activity of SHP-1 or deletion of \textit{Ptpn6} in DP thymocytes alleviated the developmental block imposed by THEMIS deficiency; these results identify enhanced PTP activity of SHP-1 as the underlying cause of the maturational defect in \textit{Themis} \(^{-/}\) thymocytes. It is also

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**Figure 7** Attenuated TCR signaling responses in \textit{Themis} \(^{-/-}\) thymocytes in the presence of ROS. (\(a,b\)) Immunoblot analysis of ZAP-70 phosphorylated at Tyr319 (p-ZAP-70(Y319)) and total ZAP-70 (left margins) in \textit{Themis} \(^{+/-}\) and \textit{Themis} \(^{-/-}\) total thymocytes stimulated with various concentrations (above lanes) of concanavalin A (ConA) (\(a\)) or with various concentrations (above lanes) of H\(_2\)O\(_2\) in the presence (+) or absence (−) of anti-CD3 plus anti-CD4 (\(b\)). (\(c\)) Immunoblot analysis of signaling responses (left margin) in \textit{Themis} \(^{+/-}\) or \textit{Themis} \(^{-/-}\) total thymocytes cultured for 0 h (left) or 6 h (right) at 37°C in serum-free medium and left unstimulated (0) or stimulated for 5 or 15 min (above lanes) with anti-CD3 plus anti-CD4, assessed as tyrosine phosphorylation (pY), ZAP-70 phosphorylated at Tyr394 (p-ZAP-70(Y394)) and total ZAP-70, and total THEMIS (left margin). (\(d\)) Immunoblot analysis of signaling responses (left margin; as in \(c\)) in total thymocytes obtained from mice with transgenic expression of THEMIS, or \textit{Themis} \(^{+/-}\) or \textit{Themis} \(^{-/-}\) mice, then cultured for 6 h at 37°C in serum-free medium and left unstimulated (0) or stimulated for 2, 5 or 10 min (above lanes) with anti-CD3 plus anti-CD4. (\(e\)) Immunoblot analysis of tyrosine-phosphorylated and total ZAP-70 (as in \(c\)) in \textit{Themis} \(^{+/-}\) or \textit{Themis} \(^{-/-}\) total thymocytes treated with various concentrations (above lanes) of H\(_2\)O\(_2\). (\(f\)) Immunoblot analysis of tyrosine-phosphorylated and total ZAP-70 and SHP-1 (as in \(c\)), and total LCK and THEMIS (left margin), in HEK-293 cells transfected with various combinations (above lanes) of plasmids encoding LCK (always included, to induce phosphorylation of ZAP-70), ZAP-70, THEMIS and/or SHP-1. (\(g\)) Immunoblot analysis of SYK phosphorylated at Tyr352 (p-SYK(Y352)) and total SYK, tyrosine-phosphorylated and total SHP-1 (as in \(c\)) and total THEMIS in HEK-293 cells transfected with various combinations (above lanes) of plasmids encoding SYK, THEMIS and SHP-1. Data are representative of three experiments (\(a,b,d,e\), four experiments (\(c\)) or two experiments (\(f,g\)).
relevant that T cell development is not ‘rescued’ in either Themis−/− mice deficient in BIM, a proapoptotic member of the BCL-2 family of prosurvival proteins (Bim−/−) or Themis−/− mice with transgenic expression of BCL-2 (ref. 7), which indicates that the developmental block in Themis−/− thymocytes is not secondary to increased negative selection, as has been speculated.

It is well established that DP thymocytes are more sensitive to TCR stimulation than are mature T cells19. Although the mechanism(s) underlying this sensitivity has (have) remained unclear, the enhanced signaling ability 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 might be responsible for the selective sensitivity of DP thymocytes to engagement of the TCR. Consistent with this, DP thymocytes have high expression of THEMIS, but its expression is downregulated as thymocytes transition to the SP stage and become less responsive to low-affinity self ligands7, a property that is necessary for the prevention of autoimmunity. Thus, stage-specific regulation of THEMIS represents a mechanism for transient and selective attenuation of SHP-1 activity in DP thymocytes to enable positive selection while preserving the SHP-1-mediated inhibitory pathway for limiting the responsiveness of mature T cells.

Our results suggest that in addition to regulating SHP-1, THEMIS might also regulate the activity of SHP-2, a closely related class I SH2 domain PTP. THEMIS bound to SHP-2, and the PTP activity of SHP-2 was attenuated by THEMIS (albeit only slightly in vitro assays). If THEMIS does have an inhibitory effect on SHP-2 in thymocytes, this might help to explain the observation that under certain stimulatory conditions, the kinase ERK and calcium signaling responses to engagement of the TCR are enhanced in Themis−/− thymocytes16. Unlike SHP-1, which is thought to have an exclusively inhibitory role in signal transduction, SHP-2 positively regulates ERK and calcium-NFAT–mediated signaling6,43,44. However, it is notable that in contrast to the ‘rescue’ observed in Themis−/−Ptprn6−/− mice, deletion of the gene encoding SHP-2 (Ptpn11) did not alleviate the block in positive selection in Themis−/− mice (data not shown), which would indicate that the main function of THEMIS in thymocytes is to regulate the catalytic activity of SHP-1.

Our results have also identified an important role for ROS in thymocyte selection. Published data have suggested that ROS might have a role in positive selection and the maturation of SP thymocytes, but this mechanism has not been investigated extensively45,46. ROS, generated following the activation of T cells 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 the activation and effector responses of mature T cells, in part by inhibition of SHP-1 (ref. 47). The origin of ROS production in the thymus remains to be elucidated and could include both intrinsic (thymocyte-derived) sources and extrinsic (cortical epithelial, dendritic cell or macrophage) sources. The defects in TCR signaling in Themis−/− thymocytes, which stem at least in part from diminished oxidative inactivation of SHP-1 by ROS, are most clearly revealed under conditions in which 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 by studies in which activation of Themis−/− thymocyte was performed under conditions in which ROS are not present or are not produced7,8,14,16.

Analysis of a comprehensive collection of over 100 species across the eukaryotic tree has revealed that the CABIT module is found only in metazoan and that its emergence correlates with the expansion of the phosphorylated-tyrosine signaling network9 (data not shown). Together with the biochemical function established for THEMIS in our study here, this suggests that the CABIT module evolved in metazoan as a mechanism for regulating phosphorylated-tyrosine signaling. The sequence diversity outside the core sequence of CABIT modules raises the possibility that different CABIT modules might have evolved to interact with distinct PTPs. That, combined with organ-restricted or developmentally restricted expression of CABIT proteins, such as that 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.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.C., C.W., E.L., J.L. and J.A. performed the experiments; S.C., R.L. and P.E.L. were responsible for the concept and experimental design; L.A. performed proteomics, protein modeling and evolutionary analysis for the CABIT module and CABIT proteins; and P.E.L. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. *Thermis*+/− mice and mice with transgenic expression of THEMIS13 were generated as described. Ptpn6flox/flox mice were obtained from M. Muschen (UCSF). *Grb2flox/flox mice* were obtained from H. Gu (McGill University). LCK-Cre and CD4-Cre transgenic mice13 were obtained from Taconic. All animal experiments were performed according to ACUC approved protocols (ASP# 15-020; PEL).

Antibodies and reagents. For stimulation, biotin-anti-CD3 (553060), biotin-anti-CD4 (553728), and biotin-anti-CD28 (553296), used at 0.5 µg each per 1 × 10⁷ thymocytes, were from BD Biosciences. For immunoprecipitation, anti-Flag (F1804) Sigma-Aldrich; anti-SHP-1 (SC287) and anti-SHP-2 (SC280), with 1 µg of antibody used for 1 × 10⁷ thymocytes, were from Santa Cruz. For immunoblot analysis, anti-pTyr (05321), anti-GST (06332), anti-ERK (06182), anti-SHP-1 (06117) EMD Millipore; anti-pTyr564-SHP-1 (8849) and anti-pTyr416-Src (2101) were from Cell Signaling Technology; anti-pTyr319-ZAP-70 (612574) and anti-GRB2 (610111) were from BD Biosciences; anti-LCK (SC433), anti-SYK (SC1077), anti-ZAP-70 (SC574), anti-PEK (SC7383) and anti-hemaggulatin tag (SC8055) were from Santa Cruz; anti-PTPN7 (ab118978) was from Abcam; anti-Myc tag (M0473) was from MBL International; anti-SHP-1 (MS1190) was from Thermo Fisher; anti-pTyr536-SHP-1 (SP1571) was from ECM Biosciences; anti-Akt (A5414) was from Sigma-Aldrich; and anti-oxidized PTP active site mAb (MAB2844) was from R&D Systems. The antibodies for immunoblot analysis were diluted 1:1,000. The rabbit polyclonal antiserum to THEMIS7 or THEMIS2 (ref. 13) has 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. GST-THEMIS was subcloned into pEGV vector by PCR. THEMIS (C153A and C413A) was generated by site-directed mutagenesis with the Quik Change Kit (Stratagene). FLAG-tagged THEMIS2 was subcloned into pFLAG-CMV2 vector by PCR with mouse cDNA for THEMIS2 (ref. 49). PTPN7 cDNA was provided by L. Tautz (Sanford-Burnham Medical Research Institute). Plasmid encoding hemagglutinin-tagged PTPN1 was a gift from J. Chernoff (Fox Chase Cancer Center).

Immunoprecipitation and immunoblot 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, were 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 Na₃VO₄, 10 mM NaF, and protease inhibitors (Roche)). Immunoprecipitation and immunoblot analysis were performed as described14.

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 × 10⁶ cells were co-transfected with the appropriate plasmid using Lipofectamine 2000 (Thermo Scientific). For perivanadate treatment, cells were first serum starved for 16 h after transfection.

GST-precipitation assays. GST–SHP-1 protein was purchased from Abcam. His-tagged THEMIS protein was purified by Ni-NTA column from the transformed *Escherichia 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 Na₃VO₄, 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 and 1 mM Na₃VO₄ in 1× 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 (RRRLEDAEpyYARG) was added at a concentration of 0.2 mM in phosphate 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 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 µM 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 H₂O₂. Thymocytes or HEK-293 cells were stimulated with pervanadate for 10 min or H₂O₂ for 5 min at room temperature. Pervanadate (1 mM stock) was prepared with 1 mM Na₃VO₄ mixed with 5 mM H₂O₂ (final concentration) was 0.5–5 mM as noted for individual experiments. Following treatment, cells were washed with degassed 1× PBS and lysed in degassed standard lysis buffer including 10 mM iodoacetamide and 10 mM NEM. Immunoblot analysis of SHP-1 oxidation was performed with antibody to oxidized PTP active site.

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 Na₃VO₄ and washed with degassed 1× GST wash buffer minus Na₃VO₄. Beads were incubated with or without His-THEMIS fusion protein in phosphate assay buffer for 10 min on ice and then perivanadate was added and protein solution was incubated for 10 min at room temperature. Reactions were washed with degassed GST wash buffer minus Na₃VO₄. SHP-1 oxidation was visualized by immunoblot analysis with antibody to oxidized PTP active site.

In vitro thymocyte-differentiation assay. The in vitro thymocyte differentiation assay was performed as described23. In brief, DP thymocytes purified by magnetic bead enrichment (Miltenyi) were reseeded in RPMI 1640 (supplemented with 50 µM 2-mercaptoethanol and 10% charcoal/dextran treated FBS) and incubated overnight in wells coated with anti-CD3 (10 µg/ml) plus anti-CD2 (5 µg/ml; RM2-5; BD Biosciences). Cells were extensively washed and either analyzed immediately by flow cytometry (stimulatory culture) or incubated for 24 h in the same medium before analysis by flow cytometry (recovery culture). Sodium stibogluconate (CAS# 15-020; PEL) 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).

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

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**Corrigendum**: Quantifying the shifting landscape of B cell immunodominance

Gordon A Dale, Jessica R Shartouny & Joshy Jacob

*Nat. Immunol.* 18, 367–368 (2017); published online 22 March 2017; corrected after print 3 April 2017

In the version of this article initially published, a spelling error was made in the citation list. The error has been corrected in the HTML and PDF versions of the article.

**Erratum**: Epigenetic landscapes reveal transcription factors that regulate CD8\(^+\) T cell differentiation

Bingfei Yu, Kai Zhang, J Justin Milner, Clara Toma, Runqiang Chen, James P Scott-Browne, Renata M Pereira, Shane Crotty, John T Chang, Matthew E Pipkin, Wei Wang & Ananda W Goldrath

*Nat. Immunol.*; doi:10.1038/ni.3706; corrected online 27 March 2017

In the version of this article initially published online, some labels in Figure 2 were illegible or incorrect. Those should read "Enhancers (× 10\(^3\))" along the top and "TE, MP and M" (top to bottom) along the left margin of Figure 2a; "N, TE, MP and M" (left to right) above the plot in Figure 2b; and "GO" below the plot in Figure 2c. Also, in the third sentence of the final paragraph of the final subsection of Results (Validation of PageRank-predicted TFs), the description of the control cells ("shCon-transfected") was incorrect. The correct text is "...lower among shNr3c1-transduced cells than among shCon-transduced cells...". The errors have been corrected in the print, PDF and HTML versions of this article.

**Erratum**: THEMIS enhances TCR signaling and enables positive selection by selective inhibition of the phosphatase SHP-1

Seeyoung Choi, Claude Warzecha, Ekaterina Zvezdova, Jan Lee, Jérémy Argenty, Renaud Lesourne, L Aravind & Paul E Love

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In the version of this article initially published online, in the second sentence of the first paragraph of the third subsection of Results (‘Deletion of *Ptpn6* restores T cell development in *Themis*\(^−/−\) mice’), the TCR chain is identified incorrectly as ‘CD3’; that phrase should read ‘...antibody to the TCR invariant chain CD3\(\varepsilon\) (anti-CD3\(\varepsilon\))...’ instead. In the legend to Figure 3, the \(P\) value (\(< 0.005\)) was incorrect; the correct value is \(< 0.05\). Also, in the final sentence of that legend, the directions ‘(left)’ and ‘(right)’ are incorrect; that should read ‘Data are representative of (top) or from (bottom) four experiments...’ instead. In Figure 4a, the numbers along the horizontal axes are incorrectly vertical; they should be horizontal instead. In Figure 4b, the labels along the vertical axes of the second and fourth plots incorrectly include ‘(%)’; the correct label is ‘CD8SP cells (×10\(^6\))’ only. In the third sentence of the first paragraph of the fifth subsection of Results (‘p-SHP-1 does not correspond with PTP activity’), the word ‘of’ is missing; this should read ‘The lower abundance of p-SHP-1...’ instead. In the legend to Figure 5c, the antibody is incorrectly set off in commas; that should read ‘...immunoprecipitated with anti-SHP-1 from...’ instead. Finally, Figure 7e is too large and should be the same size as all other panels in that figure. The errors have been corrected in the print, PDF and HTML versions of this article.

**Erratum**: CCL19-CCR7–dependent reverse transendothelial migration of myeloid cells clears *Chlamydia muridarum* from the arterial intima

Mark Roufael, Eric Gracey, Allan Siu, Su-Ning Zhu, Andrew Lau, Hisham Ibrahim, Marwan Althagafi, Kelly Tai, Sharon J Hyduk, Kateryna O Cybulsky, Sherine Ensan, Angela Li, Rickvinder Besla, Henry M Becker, Haiyan Xiao, Sanjiv A Luther, Robert D Inman, Clinton S Robbins, Jenny Jongstra-Bilen & Myron I Cybulsky

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In the version of this article initially published, the label along the horizontal axis of the graph in Figure 1a (‘Dose (mg)’) is incorrect. The correct label is ‘Dose (µg)’. The error has been corrected in the HTML and PDF versions of the article.