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Negative times negative equals positive, THEMIS sets the rule on thymic selection and peripheral T cell responses

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ABSTRACT

The activity of T cells is finely controlled by a set of negative regulators of T-cell antigen receptor (TCR)-mediated signaling. However, how those negative regulators are themselves controlled to prevent ineffective TCR-mediated responses remain poorly understood. Thymocyte-expressed molecule involved in selection (THEMIS) has been characterized over a decade ago as an important player of T cell development. Although the molecular function of THEMIS has long remained puzzling and subject to controversies, latest investigations suggest that THEMIS stimulates TCR-mediated signaling by repressing the tyrosine phosphatases SHP-1 and SHP-2 which exert regulatory function on T cell activation. Recent evidences also point to a role for THEMIS in peripheral T cells beyond its role on thymic selection. Here, we present an overview of the past research on THEMIS in the context of T cell development and peripheral T cell function and discuss the possible implication of THEMIS-based mechanisms on TCR-dependent and independent signaling outcomes.

T cells operate together with B cells to coordinate the cellular and humoral arms of adaptive immunity. The development, activation and differentiation of T cells into effector cells is controlled by a complex array of signaling events triggered by the multi-subunit T cell antigen receptor (TCR), which recognize antigenic peptides bound to MHC molecules (pMHC) presented by antigen presenting cells (APCs) [Box 1]. Unlike most cell surface receptors that recognize a single ligand, individual TCRs are capable of recognizing different ligands (for example, self-peptides and foreign peptides) with a broad range of affinities. The strength of TCR-ligand interactions has a critical influence on the outcome of T cell responses. In the thymus, positive and negative selection of thymocytes is engaged to optimize T cell responses to foreign antigens and to prevent autoimmune reactions against self-antigens. Negative selection eliminates T cells

The main steps of TCR signaling

T cells express a clonotypic multi-subunit antigen receptor (TCR) which is composed of a heterodimer of α and β chain, forming the recognition subunit, that is non-covalently associated with multiple signal transducing subunits, including CD3γ, CD3δ and CD3ε and, in most cases, a CD3ζ chain. The recognition of cognate pMHC by the TCR induces conformational changes in the CD3ε subunit which enable the recruitment of the SRC family kinase member LCK which phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3γ chain, CD3δ chain, CD3ε chains and the ζ-chains. Phosphorylated ITAMs serve as docking site for the kinase ZAP70 (ζ-chain associated protein kinase of 70 kDa), which is activated following its phosphorylation by LCK. Activated ZAP70 phosphorylates four key tyrosine residues on linker for activation of T cells (LAT), which recruits several signaling complexes that seed distinct signaling pathways. Those proteins include the growth factor receptor-bound protein 2 (GRB2), which recruit several proteins such as THEMIS, VAV1, SHIP1 and the Ras-GTP exchange factor SOS1, the GRB2-related adaptor protein GADS and the adaptor protein SLP76 (SH2 domain-containing leukocyte protein of 76 kDa), which enables the recruitment of adhesion- and degranulation-promoting adaptor protein (ADAP), interleukin-2-inducible T cell kinase (ITK), NCK1 and VAV1 and the phospholipase Cγ1 (PLCγ1). Those complexes trigger several signaling pathways among which the best characterized are the Ca^{2+} flux which lead to the translocation of the transcription factor NFAT in the nucleus, the mitogen-activated protein kinase (MAPK) kinase which lead to the activation of transcription factors such as AP-1 and the nuclear factor-κB (NF-κB) signaling pathways which induce the activation and the translocation in the nucleus of transcription factors such as c-REL and RELA.

that strongly interact with self-pMHC molecules, which could otherwise result in autoimmunity, whereas positive selection promotes the survival and maturation of T cells with relatively low affinity for self-pMHC molecules, favoring the development of cells with greater self-reactivity within a permissible range constrained by the negative selection threshold [1–3]. In the periphery, stimulation of the TCR by low affinity antigens promotes signals that favor their maintenance, whereas stimulation by higher affinity ligands may promote full activation leading to clonal expansion and differentiation into effector subsets.

The magnitude and the type of signaling pathways induced following antigen stimulation is primarily set by the strength of interaction between the antigen and the TCR. They also depend on regulatory proteins, which are not directly involved in the propagation of signaling events but which contribute to regulate their magnitude and their localization [4]. Although many regulators of TCR signaling have now been identified, their precise functions in controlling *in vivo* TCR-mediated developmental or effector responses have rarely been formally demonstrated. Many of these proteins show variable expression profile throughout development and in effector subsets indicating that they contribute to adjust the sensitivity of T cells to antigen stimulation in given immunological contexts. In the thymus for instance, thymocyte responsiveness to self-ligand is enhanced by a set of positive regulators of TCR signaling which are up-regulated in CD4+CD8+ double-positive [DP] thymocytes prior the stage of positive selection [4]. In the periphery, negative regulators of TCR signaling are enhanced in specific subsets, reducing the reactivity of immune cells to self-antigens and preventing exacerbated inflammatory responses, which may cause pathology [4].

Thymocyte-expressed molecule involved in selection-1 (THEMIS; also referred to as THEMIS1) is a prototypical example of regulatory proteins involved in the tuning of T cell activation. THEMIS was identified almost concomitantly by five research groups in 2009 through approaches aiming at screening for T cell phenotypes in N-ethyl-N-nitrosourea

(ENU)-induced mutant mice and by genetic or bioinformatics screening for genes specifically expressed in thymocytes [5–9]. Each studied independently reported a comparable phenotype in THEMIS deficient mice, characterized by a reduced maturation of DP cells into CD4+ and CD8+ single-positive [SP] with a more severe reduction in CD4SP thymocytes and peripheral T cells. The characterization of THEMIS also led to the identification of two related mammalian homologues, Icb-1 (subsequently renamed THEMIS2), which is expressed in B and myeloid cells, and THEMIS3, which is expressed only in the intestine [5,6,8]. Four groups also reported the interaction of THEMIS with the Growth factor receptor-bound protein 2 (GRB2), an adapter protein which serves as a molecular intermediate for the recruitment of signaling proteins to transmembrane proteins and receptors [6–9]. Despite the striking block of T cell development observed in THEMIS deficient mice and several indirect evidences suggesting positive regulatory function of THEMIS on TCR signaling, it is only a decade after its initial characterization and some controversies that the mechanism by which THEMIS regulates TCR signaling began to be elucidated [10]. The difficulty for resolving the function of THEMIS proteins have relied partly on the unusual and apparently "non-ergonomic" mechanism by which THEMIS stimulates TCR signaling, which is based on the inhibition of the catalytic activity of the SH2-domain containing tyrosine phosphatases SHP-1, a protein which exerts an inhibitory function on the signaling events triggered by several T-cell expressed receptors including the TCR. A stimulatory mechanism based on the inhibition of inhibitory proteins may seem counterintuitive and unnecessarily "costly" energetically speaking. Nevertheless, it possibly allows to put negative regulators of T cell activation under control, in a flexible and precise way, maintaining those proteins available for repressing T cell activity if rapidly needed. Here, we present an overview of the past research on THEMIS in the context of T cell development and peripheral T cell function and propose different interpretations to explain previous contrasting results associated to the function of this protein.

Structure and molecular function of THEMIS during T cell development

THEMIS1 is the founding member of a metazoan protein family containing CABIT modules

THEMIS proteins do not contain any known domains with catalytic function. Only two motifs are present in the three members of the THEMIS protein family, a C-terminal proline-rich sequence (PRS) composed of two proline rich motifs (PRR1 and PRR2) and a centrally located nuclear localization signal. Among the five groups which initially characterized THEMIS, one analyzed the amino-acid sequence of THEMIS with a program that uses empirically determined entropy thresholds to identify potential globular domains [6]. This analysis led to the identification of two putative tandem repeat globular domains of approximately 260 amino-acid length that they designated cysteine-containing all-beta-in-THEMIS (CABIT) module. These modules are predicted to rearrange in an SH3-like β-barrel fold which contain a conserved core sequence (wXCX7−26wXLPwX3GXF with X = any amino acid and w = any hydrophobic residue) and several conserved residues, among which a highly conserved cysteine, predicted to form ligand-binding contact sites. Further analysis of database identified hundreds of uncharacterized proteins in metazoan with the same globular domain [6]. In mammals, two proteins containing a single CABIT-module were identified in addition to the three THEMIS homologues: GAREM1 which is broadly expressed [11], and GAREM2, which is expressed in neuronal cells [12]. GAREM1 and GAREM2 as well as other proteins containing single-CABIT module contain also a PRS which is combined frequently with a C-terminal SAM (sterile a-motif) domain. The signaling function of THEMIS CABIT domains has been resolved in a recent study [13], however how they biochemically operate and whether they exert catalytic activity remains unclear.

THEMIS CABIT domains inhibit the tyrosine-phosphatase activity of SHP-1 and SHP-2

Quantitative analysis of THEMIS interacting partners by mass spectrometry in Jurkat cells and thymocytes identified the protein tyrosine phosphatase (PTP) Src homology region 2 domain-containing phosphatase-1 and -2 (SHP-1 and SHP-2) among the preferential binding partners of THEMIS, with SHP-1 interaction largely predominating over SHP-2 [14]. These phosphatases are well characterized regulators of T-cell signaling which operate downstream of several receptors including the TCR. SHP-1 is described almost exclusively as a negative regulator of T cell activation and differentiation. It interacts with proteins of the TCR signaling machinery and has been proposed to operate within negative feedback loops that contribute to negatively regulate TCR signaling upon recognition of weak and strong agonists by the TCR [15,16]. SHP-1 is also directly recruited to inhibitory receptors containing immunoreceptor tyrosine-based inhibitory motifs (ITIM) and immunoreceptor tyrosine-based switch motifs (ITSM) which are phosphorylated upon stimulation by their respective ligands [17−20]. The presence of dually phosphorylated motifs on the cytoplasmic tail of these receptors enables the binding in cis of the two tandem SH2 domains of SHP-1, which contributes to stabilize SHP-1 at the membrane, in proximity of its signaling targets, and to enhance its activity by interrupting an intramolecular association between the N-terminal SH2 domain and the PTP domain of SHP-1 [21,22]. Several potential substrates of SHP-1 have been identified in lymphocytes, including tyrosine residues located on the activation sites of proteins such as the tyrosine kinases LCK and ZAP-70 [23,24], which are required for the initiation of TCR signaling, the adapter proteins LAT and SLP-76 [25,26], and the guanine nucleotide exchange factor (GEF) Rho family guanosine triphosphatases (GTPase) VAV1 [27,28]. The role of SHP-2 on TCR signaling appears to be more complex, with both inhibitory and activating roles being ascribed depending on the substrate and the context of its recruitment to the membrane. Whereas SHP-2 is described as a negative regulator of TCR- and CD28-mediated signaling events following its recruitment to inhibitory receptors such as the programmed cell death 1 receptor (PD-1) [29−31], SHP-2 is associated to positive function on TCR signaling when TCR signals emanate exclusively from the TCR and do not integrate additional stimulatory inputs [32,33].

In a key study, the group of Paul Love examined in details the mechanism by which THEMIS interacts with SHP-1 and SHP-2 and identified important functions for the CABIT domains in controlling the activity of these phosphatases [13]. Indeed, THEMIS was found to bind directly to SHP-1 through its CABIT modules. SHP-1 could bind to truncated THEMIS proteins encoding isolated CABIT modules but not as efficiently as with THEMIS proteins containing the tandem repeat modules. Further analysis in this study showed that the CABIT modules interact specifically with the phosphatase domain of SHP-1, suggesting that THEMIS could exert modulatory function on SHP-1 phosphatase activity. *In vitro* analysis with recombinant proteins showed that both THEMIS1 and THEMIS2 could inhibit the tyrosine phosphatase activity of SHP-1. Proteins encoding only for the tandem repeat CABIT modules were as efficient as the full-length THEMIS to inhibit the phosphatase activity of SHP-1 while the isolated CABIT1 module could partially inhibit SHP-1 activity. THEMIS could also inhibit the phosphatase activity of SHP-2, although not as efficiently as SHP-1, and had no effect on other related type-1 tyrosine phosphatases such as PTPN1 and PTPN7, indicating a specific effect of THEMIS on the phosphatases from the SHP family. Investigation of the mechanism by which THEMIS controls SHP-1 activity indicated that CABIT modules operate at least partly by promoting or stabilizing the oxidation of the catalytic cysteines of SHP-1, thereby maintaining SHP-1 in an inactive form. The conserved cysteines in the core region of THEMIS CABIT modules were not required for the inhibition of SHP-1 phosphatase activity, confirming previous observations showing that those cysteines are dispensable for THEMIS activity *in vivo* [34]. It was speculated that the CABIT module may operate by preventing the access of the oxidized catalytic cysteine of SHP-1 and SHP-2 to reducing agents or by exposing the catalytic cysteine to oxidation by reactive oxygen species ROS. The ability of THEMIS to block SHP-1 phosphatase activity in cell free assay also suggests that the inhibitory effect
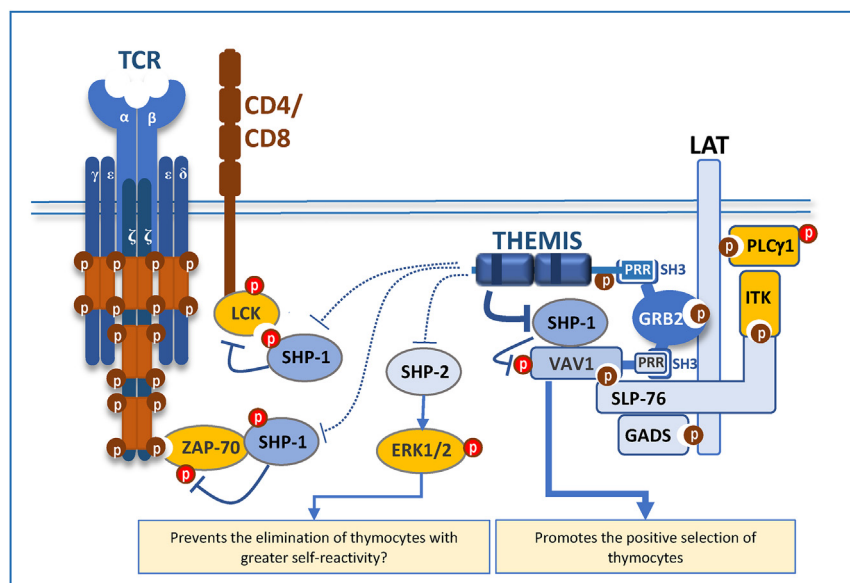
Fig. 1 THEMIS interactome and function in the context of TCR signaling. Upon TCR stimulation, THEMIS is recruited to LAT in proximity of VAV1 *via* the adapter protein GRB2. THEMIS promotes VAV1 activation and the positive selection of thymocytes by repressing the phosphatase activity of SHP-1. THEMIS also promotes the activation of LCK and ZAP-70 by repressing SHP-1, but may have a reduced influence on the activity of those proteins as compared to that for VAV1 due to increased remoteness. THEMIS concomitantly inhibits ERK1/2 activity by repressing SHP-2. This might skew the mature TCR repertoire toward greater self-reactivity and favor the development of optimized immune responses to pathogens. Signaling proteins which are most predominately enriched in THEMIS interactome in thymocytes are represented in blue. Phosphorylation events that are regulated by THEMIS are represented in red.

of THEMIS cannot solely be explained by redox regulation and that CABIT modules probably also block access of the catalytic cysteine to phosphorylated-tyrosine ligands. Since SHP-1 is a well-characterized inhibitor of TCR signaling [23,24,26,27] and since THEMIS inhibits preferentially SHP-1 over SHP-2 [13], a straightforward prediction from those findings is that THEMIS should operate as a stimulator of TCR signaling. However, analysis of TCR signaling in THEMIS deficient T cells in the past years have led to contrasting results which posit nearly opposite function of THEMIS on T cell development.

Contrasting effects of THEMIS on TCR signaling

Despite the striking phenotype of THEMIS deficient mice and the identification of interactions between THEMIS and TCR signaling proteins, detecting a modulatory effect of THEMIS on TCR signaling has been particularly challenging and has led to equivocal results. Among the five research groups that initially reported the characterization of THEMIS, four of them failed to detect defects associated to THEMIS deficiency following TCR cross-linking *in vitro* [6—9] and one observed a limited decrease of ERK phosphorylation and calcium flux which is in contrast to the fairly important block of T cell development observed in *Themis−/−* mice [5]. The positive effect of THEMIS on TCR signaling was thus initially deducted indirectly from the phenotype of THEMIS deficient mice which show several defects of T cell development all suggestive of reduced TCR signal intensity. For instance, positive selection and CD4+ T cell lineage commitment, which are dependent on sustained TCR signals, and negative selection, which

required intense TCR signals were all impaired in *Themis−/−* mice [5,6,8].

Unexpectedly, later studies identified inhibitory function for THEMIS on TCR signaling events in thymocytes and Jurkat cells stimulated *in vitro* by low-affinity antigens [35,36]. It was shown first that the loss of THEMIS results in enhanced calcium (Ca^{2+}) flux and increased phosphorylation of LCK, LAT, and ERK in preselected DP thymocytes that are stimulated by low-affinity pMHCs tetramers [35]. In that study, which was published before CABIT modules were reported to exert inhibitory function on SHP-1 phosphatase activity, THEMIS was speculated to function by facilitating the recruitment of SHP-1 and SHP-2 to the transmembrane adaptor LAT where they would be brought into contact with their primary targets. It was hypothesized here that THEMIS operates by preventing the transmission of strong signaling events that might be inadequately triggered by low-affinity self-ligands possibly leading to misplaced negative selection. However, arguing against this model, the disruption of *Bcl2l11*, the gene encoding BIM, a pro apoptotic factor required for negative selection [14], or the overexpression of BCL2 [8] could not optimally rescue positive selection of *Themis−/−* thymocytes. In addition, analysis of TCR signaling in developing thymocytes using the transgenic TCR signaling reporter system (Nur77-GFP) indicated positive effect of THEMIS on TCR signaling in response to endogenous selecting ligands [14]. Finally, and probably most importantly, the deletion of *ptpn6*, the gene encoding for SHP-1, could alleviate the developmental block in *Themis−/−* thymocytes [13], suggesting that the impaired maturation of SP thymocytes observed in *Themis−/−* results

from an exacerbated inhibitory activity of SHP-1 on TCR signaling rather than from an attenuated function of this phosphatase. Altogether, a puzzling conclusion from those studies is that the loss of THEMIS would result in different outcomes on TCR signaling according to the context of stimulation, leading to exacerbated TCR signaling events when TCR signals emanate exclusively from the TCR (situation observed *in vitro* upon pMHC tetramer stimulation [35]), and to reduced TCR signal intensity when it integrates multiple regulatory inputs triggered by additional environmental stimuli [13,14].

THEMIS drives opposite effects on TCR signals that might serve different purposes on selection

One potential explanation to those observations may relate to the differential contribution of SHP-1 and SHP-2 to the regulation of TCR signaling. Although an inhibitory function on TCR signaling has been ascribed to SHP-2 [19,29–31], when recruited to ITIM-containing receptors, positive effect of SHP-2 on TCR signaling have also been reported, particularly when TCR signals emanate exclusively from the TCR [32,33,37]. Inactivation of SHP-2 catalytic function in Jurkat cells [33] and conditional disruption of *Ptpn11*, the gene encoding SHP-2, in T cells [32] result in impaired activation of the MAP kinases ERK1/2 following TCR cross-linking which is associated to the inefficient maturation of thymocytes after the β-selection checkpoint. SHP-2 also promotes the activation of SRC kinases by dephosphorylating the adaptor protein CBP/PAG, which prevents the recruitment to this adaptor of the C-terminal Src kinase (CSK) that phosphorylate SRC kinases on inhibitory tyrosine residues [37,38]. The reason of this dual effect of SHP-2 on TCR signaling is unknown but it contrasts with the univocal inhibitory function ascribed to SHP-1 on TCR signaling. Thus, if THEMIS represses concomitantly SHP-1 and to a lesser extent SHP-2, a prediction would be that THEMIS may exert combined effect on TCR signaling events, promoting specific TCR signals that are repressed by SHP-1 and inhibiting ones that are stimulated by SHP-2. Such dual effects of THEMIS on TCR signaling was reported in a previous study from our group in thymocytes stimulated with low or high doses of anti-CD3 antibodies [14]. The quantitative analysis of the THEMIS interactome by mass spectrometry reveals that in addition to GRB2, SHP-1 and to a lesser extent SHP-2, THEMIS also strongly interacts with the RAC1 Guanine nucleotide exchange factor VAV1, which plays an important role in T cell development and peripheral T cell functions [14,39]. We showed that the loss of THEMIS results in increased ERK phosphorylation when thymocytes are stimulated under weak TCR cross-linking conditions, supporting previous results obtained in thymocytes with low affinity ligands [35]. However, the phosphorylation of VAV1 was strongly decreased in the absence of THEMIS independently of the strength of TCR stimulation. This effect was correlated with a reduced production of RAC1-GTP a direct product of VAV1 and was also observed *ex-vivo* in post-selection thymocytes expressing a transgenic TCR [14]. Since SHP-2 exerts a positive effect on ERK1/2 activity [32,33,37] and since phosphorylated tyrosine residues on VAV1 are well-characterized substrates of SHP-1 [27,28], it could be speculated that the inhibitory

effect of THEMIS on ERK1/2 may result from its ability to block SHP-2 [13], whereas its stimulating effect on VAV1 may result from its repressing effect on SHP-1 [13]. This observation raises the question of whether this combined effect of THEMIS on TCR signaling is relevant to its *in vivo* function or whether the inhibitory effect reported for THEMIS on TCR signaling *in vitro* is marginal in regard of the stimulating effect detected *in vivo*. Knock-in mice expressing a GEF-deficient form of VAV1 exhibit a block in T cell development at the DP to SP transition, which is similar to the phenotype of *Themis−/−* mice [39], suggesting that the stimulating effect of THEMIS on VAV1 activity could by itself explain the effect of THEMIS on T cell development [Fig. 1]. Strong but transient activation of ERK, similar to what is seen in *Themis−/−* thymocytes, is coupled with negative selection [40], suggesting that THEMIS might also be important to equalize ERK-mediated signaling and prevent the transmission of negatively selecting stimuli that may lead to some form of exacerbated negative selection [Fig. 1]. Positive selection is proposed to operate by skewing the mature TCR repertoire to self and foreign pMHC toward higher affinity in order to optimize immune responses to pathogens [2]. Thus, by concomitantly enhancing VAV1 activity and repressing ERK-mediated signaling, THEMIS on one hand might enable the maturation of thymocytes capable of recognizing self-pMHC and, on the other hand, enhance the negative selection threshold to prevent the elimination of T cells which might be more effective against pathogens. Importantly, the inhibitory effect that THEMIS might exert on TCR signaling by blocking SHP-2-mediated ERK1/2 activation does not seem to account for the strong phenotype of *Themis−/−* mice since the deletion of *ptpn11*, does not alleviate the block of T cell development observed in those mice [13]. Altogether, if THEMIS inhibits signaling events associated to negative selection by blocking SHP-2, this might be marginal phenotypically speaking in regard of its stimulating effect on positive selection signals which occur by blocking SHP-1 phosphatase activity.

GRB2 regulates THEMIS-mediated SHP-1 inhibition through multiples processes

The possibility that THEMIS would belong to the TCR signaling machinery was suggested early by its interaction with GRB2, an adapter protein involved in the recruitment of signaling proteins in proximity of many cell surface receptors, including the TCR and the BCR. Analysis of the THEMIS interactome by mass spectrometry indicates that GRB2 is by far the most important binding partner of THEMIS in both thymocytes [14] and peripheral T cells [41]. Stoichiometric analysis of THEMIS interaction with GRB2 indicates that approximately half of THEMIS proteins binds to GRB2 whereas about one tenth of GRB2 binds to THEMIS in thymocytes [42]. THEMIS also binds to the cytosolic adapter proteins GRAP2, although THEMIS-GRB2 complexes are ten times more abundant than THEMIS-GRAP2 complexes [14]. GRB2 contains an SH2 domain flanked by two SH3 domains. THEMIS interacts directly with GRB2 through its first proline rich region (PRR1) which recognizes one of GRB2 SH3 domain [9,43,44]. The reconstitution of THEMIS deficient thymocytes with transgene or retroviral constructs expressing mutated forms of THEMIS deficient for

the PRS fail to restore normal T cell development, highlighting the crucial role for THEMIS-GRB2 interaction on THEMIS signaling function *in vivo* [34,42]. The role of GRB2 on THEMIS-mediated signaling is complex and encompasses several molecular functions.

GRB2 operates as a molecular bridge between THEMIS and SHP-1

It was proposed that GRB2 could operate by facilitating the interaction between THEMIS and SHP-1 [36]. Mutation of key proline residues on THEMIS PRS that disrupt THEMIS-GRB2 interaction also prevent THEMIS from interacting with SHP-1 [36]. GRB2 interacts with SHP-1 through its N-terminal SH3 domain [36] and through its SH2 domain which recognize tyrosine residues on the C-terminal tail of SHP-1 tail that are phosphorylated by the kinase LCK after TCR stimulation [45]. Since the interaction between THEMIS and SHP-1 is not affected by LCK deficiency in Jurkat cells, the N-terminal domain of GRB2 was speculated to be important for anchoring SHP-1 to THEMIS-GRB2 complexes [36]. Although two studies suggest that THEMIS binds preferentially to the N-terminal domain of GRB2 [9,43], one study reported on the contrary that THEMIS predominantly interact with its C-terminal domain *via* its PRR1 [44], suggesting that THEMIS and SHP-1 could concomitantly bind to GRB2, which would operate in this context as a bridging structure between THEMIS and SHP-1. Arguing against this possibility, analysis of THEMIS-SHP-1 interaction in GRB2 deficient thymocytes showed that GRB2 is not required for this interaction [13]. GRB2 is also dispensable for THEMIS to inhibit SHP-1 catalytic activity *in vitro* [13]. Thus, although GRB2 might assemble concomitantly with THEMIS and SHP-1 in a tri-molecular complexes, it does not seem to be required for THEMIS-SHP-1 interaction.

GRB2 concentrates THEMIS in proximity of SHP-1 and its molecular target VAV1

Another potential function of GRB2 is to promote the recruitment of THEMIS to the Linker for Activation of T cells (LAT) [43,44], a transmembrane adapter protein which contributes to signal diversification in T cells by recruiting multiple signaling complexes that constitute seeds for distinct signaling pathways. The SH2 domain of GRB2 enables its recruitment to several tyrosine-phosphorylated ligands at the cell membrane, including LAT [46] and CD28 [47]. THEMIS proteins lacking the PRS fail to relocate to the immune synapse and to be recruited to LAT following TCR stimulation [44]. The functional consequence of THEMIS recruitment to LAT is unclear since it is presumably not required for THEMIS interaction with SHP-1, which occurs *in vitro* through THEMIS CABIT modules in the absence of intermediates molecules [13]. Following its recruitment to LAT, THEMIS is rapidly phosphorylated by the tyrosine kinase LCK on two tyrosine residues (Y540 and Y541 in human THEMIS and Y542 and Y543 in mouse THEMIS), suggesting that the recruitment of THEMIS to LAT might be important to stimulate THEMIS-mediated signaling function through phosphorylation-based events [44,48]. Supporting this possibility, the reconstitution of THEMIS deficient bone marrow cells with lentiviral particles encoding for THEMIS proteins containing point mutations of those residues fail to restore normal T cell development in

chimeric mouse experiments [44]. However, the phosphorylation of those residues was also shown to be important for the stabilization of THEMIS-GRB2 complexes suggesting that the ineffectiveness of those mutants might simply be the consequence of altered GRB2-THEMIS interaction [44]. Several substrates of SHP-1 have been characterized. While some (such as LCK and ZAP-70) associate to the TCR and are required early in the signaling cascades to initiate TCR signaling, others associate to LAT (such as SLP-76 and VAV1) and operate later to seed distinct pathways leading to specific functional outcomes. Thus, the binding to GRB2 may ensure that THEMIS is positioned more selectively to affect the activity of the cellular pool of SHP-1, which is proximity of some of its molecular targets in the LAT signalosome. Accordingly, LCK and ZAP-70 are not enriched in the THEMIS interactome as opposed to LAT, VAV1 and to a lesser extent SLP-76 [14]. Reciprocally, THEMIS is not detected in ZAP-70 and LCK interactomes in CD4+ T cells whereas it is identified as a binding partner of LAT and VAV1 in those cells [41,49,50]. THEMIS positively regulates VAV1 phosphorylation following TCR stimulation in thymocytes but has no detectable effect on SLP-76 [14], suggesting that a potential function of GRB2 could be to bring THEMIS in proximity of VAV1 to "protect" VAV1 from SHP-1-mediated dephosphorylation. VAV1 binds to one of GRB2 SH3 domains through an unconventional PRR region located inside the SH3 domain of VAV1, supporting the possibility that a tri-molecular complex between THEMIS, GRB2 and VAV1 might form around LAT [51]. Altogether, this suggests that rather than acting broadly on SHP-1 by decreasing the level of active phosphatases at the cellular level, THEMIS
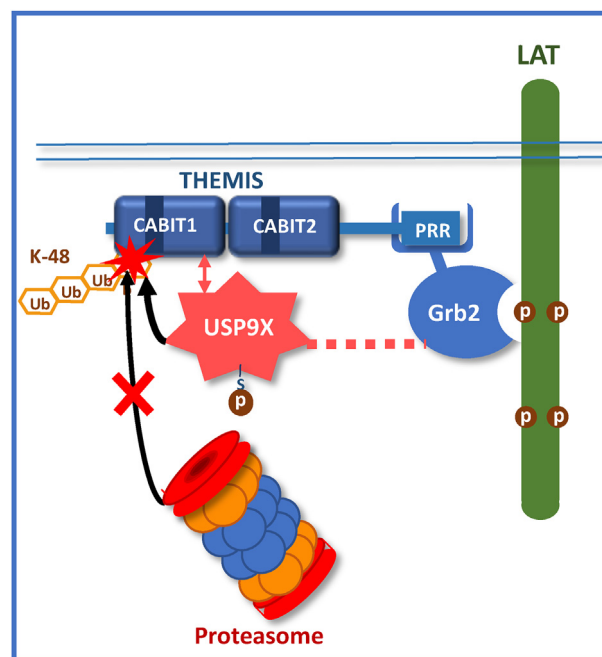

Fig. 2 GRB2 prevents proteasome-mediated degradation of THEMIS by promoting USP9X activity. The recruitment of USP9X to LAT through GRB2 and THEMIS leads to the phosphorylation of an activating serine residue on USP9X. USP9X stabilizes THEMIS expression upon TCR stimulation by removing K48-bound ubiquitin chains on THEMIS.

might operate more selectively by repressing the SHP-1-VAV1 signaling module associated to the regulation of specific cellular outcomes, such as positive selection in thymocytes. This is in agreement with recent studies discussed hereafter in this review highlighting the cooperative link between THEMIS, SHP-1 and VAV1 in the suppressive function of Treg cells [52]. On a theological point of view, this might explain why a mechanism that would repress SHP-1 at the signaling level might not be equivalent to a mechanism that would simply decrease the expression level of SHP-1.

GRB2 and THEMIS reciprocally protect each other from proteasome-mediated degradation

Recent work also identified an unexpected role for GRB2 in the control of THEMIS protein expression and stability [53]. THEMIS protein expression is increased in thymocytes undergoing positive selection, despite the strong decrease of THEMIS mRNA levels in those subsets, indicative of a post-translational control of THEMIS expression at this stage of T cell development [53]. Analysis of THEMIS in unstimulated thymocytes and in Jurkat cells transfected with cDNA encoding for tagged versions of THEMIS and ubiquitin showed that THEMIS is polyubiquitylated by K48 chain which operates by targeting THEMIS to proteasome degradation [Fig. 2]. The amount of ubiquitinylated THEMIS decreases after TCR cross-linking in thymocytes and peripheral CD4+ T cells, suggesting that THEMIS could be deubiquitnylated following TCR engagement. Mass spectrometry analysis of proteins that precipitate together with THEMIS in thymocytes led to identify several Ubiquitin-specific proteases (USP9X, USP24, USP19, and USP15), which are characterized for their ability to remove mono- or poly-ubiquitin chains on proteins [53]. Incubation of thymocytes with a pan-inhibitor of deubiquitinylating enzymes (DUBs) results in a rapid and dramatic decrease in the amount of THEMIS protein, whereas the amount of other signaling proteins is not affected. A more specific focus on the enzyme USP9X showed that it directly interacts with THEMIS and that the CABIT1 module is important for this interaction [Fig. 2]. THEMIS ubiquitinylation is up-regulated in *Usp9X−/−* thymocytes and is associated with a decreased expression of THEMIS proteins while THEMIS mRNA level remains unchanged. The recruitment of USP9X to LAT promotes its phosphorylation on one of its serine residues (S1600) and enhances its catalytic activity [54] [Fig. 2]. The interaction of USP9X with LAT was decreased following TCR engagement in thymocytes that were partially deficient for GRB2 (*Grb2+/−* mice), suggesting that GRB2-THEMIS complexes serve as intermediates for the recruitment of USP9X to LAT [53]. The ubiquitinylation of THEMIS was exacerbated in *Grb2+/−* thymocytes and correlated with a decreased expression of THEMIS in DP and SP thymocytes. Altogether those data indicate that GRB2 plays an indirect role in the stabilization of the fraction of THEMIS which is recruited to LAT following TCR engagement and operates by enabling the phosphorylation of USP9X which leads to its activation [Fig. 2]. This mechanism might favor the maintenance over time of THEMIS-mediated regulatory effect on TCR signaling and may explain early findings suggesting a role for THEMIS in sustaining TCR signals long enough to promote positive selection and commitment of DP thymocytes to the

CD4+ lineage [8]. Interestingly, a reciprocal effect of THEMIS over GRB2 stability was identified in thymocytes. GRB2 protein expression is reduced by almost two-fold in the absence of THEMIS which correlates with an enhanced ubiquitinylation of GRB2 [14]. Although the mechanism by which THEMIS controls GRB2 was not characterized, this effect seems to account at least for part of the defect observed in *Themis−/−* mice since the partial decrease of GRB2 protein expression in thymocytes (using Grb2 hemi-deficient mice) to a level that is comparable to that in *Themis−/−* thymocytes results in a partial decrease of positive selection and to lower VAV1 phosphorylation level [14]. Moreover, THEMIS transgenic expression, leading to its overexpression in thymocytes and peripheral T cells, could enhance GRB2 expression in *Grb2+/−* thymocytes. This could restore normal VAV1 phosphorylation level in *Grb2+/−* thymocytes and efficient positive selection in *Grb2+/−* mice, providing further evidences of the positive effect of THEMIS on TCR signaling during T cell development [14]. However, the modest effect of GRB2 hemi-deficiency on T cell development in comparison to the marked defect observed in *Themis−/−* mice implies that the effect of THEMIS on SHP-1 accounts for most of the defect in T cell development observed in *Themis−/−* mice.

Functions of THEMIS in peripheral T cells

One interesting characteristic of THEMIS is that its level of expression is highly variable according to T-cell subsets. THEMIS is expressed in pre-selection DP thymocytes and is further increased at early stages of positive selection in DP thymocytes expressing activation markers [53]. Its expression is then down-regulated as thymocytes mature to the SP stage and is decreased by a factor of two to three in peripheral T cells as compared to that in DP thymocytes, with two-fold higher amount in naïve CD8+ T cells than in naïve CD4+ T cells [53]. THEMIS is expressed at relatively low level in regulatory T cells (Tregs) as compared to that in conventional CD4+ T cells (four-folds decrease) [55] and is highly up-regulated in Th1 cells (seven-fold increase compared to that in naïve CD4+ T cells) [56]. The variable amount of THEMIS in T-cell subsets might be important to set distinct thresholds for T-cell activation or to amplify specific effector responses in a given immunological context. So far, little is known about the function of THEMIS in peripheral T cells mainly because *Themis−/−* mice are lymphopenic, which may introduce bias on T-cell effector responses, and might develop compensatory mechanisms in the thymus that may altered peripheral T cell responsiveness to TCR stimulation. Except for two studies [57,58], investigations related to THEMIS function in peripheral T cells were performed using mouse models presenting germline deficiencies for THEMIS with the potential bias mentioned above.

THEMIS stimulates effector responses in CD4+ T cells

Initial studies showed that peripheral CD4+ and CD8+ T cells from *Themis−/−* mice fail to proliferate efficiently and to up-regulate activation markers in response to TCR stimulation [5]. Subsequent studies on THEMIS function in peripheral T

cells were performed using rat or mouse lines that contained spontaneous or chemically-induced mutations in the gene encoding THEMIS leading to the complete or nearly complete loss of THEMIS protein expression [52,59,60]. In both models, those mutations could recapitulate the block of T cell development which was observed in Themis−/− mice. Total and naïve CD4+ T cells isolated from a rat Lewis line, containing a frameshift mutation in the Themis gene resulting in the introduction of a premature stop codon, produce higher level of IL-4, IL-10 and IL-17 in response to TCR stimulation, suggesting inhibitory function of THEMIS on CD4+ T cell responses [52]. However, mice that contain an I23N substitution in THEMIS protein, leading to nearly complete loss of THEMIS expression, exhibit a reduced development of experimental cerebral malaria (ECM) after infection with Plasmodium berghei ANKA, which was associated with a reduced production of inflammatory cytokines including IFNγ and TNFα by CD4+ and CD8+ T cells in response to TCR stimulation [60]. These mice also fail to efficiently control bacterial load after infection with Mycobacterium tuberculosis infection [60]. More recent studies show that the proliferative defect of Themis−/− CD4+ T cells in response to TCR stimulation is associated with a decreased ability of these cells to up-regulate the expression of the insulin receptor (IR), resulting in decreased glucose uptake and aerobic glycolysis [61]. Mechanistically, THEMIS would operate by enhancing the translocation in the nucleus of the transcription factor NFAT which, according to published chromatin immunoprecipitation-sequencing and RNA-sequencing data [62], promotes the transcription of IR. Whether THEMIS acts selectively on an NFAT-dependent metabolic pathway or more broadly on early TCR signaling events, the defect of NFAT being only the consequence of earlier TCR proximal defects, was not investigated in that study. Mutational analysis show that a fraction of THEMIS is translocated to the nucleus through its nuclear localization signal [43], suggesting that THEMIS could regulate transcription factors independently of its effect on proximal TCR signaling events. In addition, in vivo analysis in that study show that THEMIS deficient naïve CD4+ T cells are less potent to induce colitis following transfer into Rag-deficient mice [61]. Numbers of CD4+ T cells producing IFNγ and IL-17 are reduced in the mesenteric lymph nodes of mice that had received Themis−/− CD4+ T cells, but not in the peripheral lymph nodes, suggesting a failure of those cells to migrate into the gut rather than to expand or differentiate into effector cells [61]. Altogether, with the consideration of potential caveats related to the use of germline deficient models, those studies suggest that THEMIS is required for the development of efficient effector functions in CD4+ T cells.

THEMIS is required for the homeostatic maintenance of CD8+ T cells

More recent studies investigate the role of THEMIS in peripheral T cells using a conditional knockout (cKO) mouse model which enables the deletion of Themis gene at late stages of T cell development, in SP thymocytes, resulting in the nearly complete loss of THEMIS expression in peripheral T cells [57,58]. THEMIS deficiency in those mice result in a 50% decrease of CD8+ T cell numbers at the periphery, whereas the numbers of CD4+ T cells were not affected, suggestive of a role for THEMIS in peripheral CD8+ T cell maintenance. Experiments showed that THEMIS is required for lymphopenia induced proliferation (LIP) and for the acquisition of an activated phenotype resulting from LIP following transfer of CD8+ T cells from into Rag deficient host [57]. THEMIS also stimulates the expansion and the effector responses of OT-1 CD8+ T cells following infection with Listeria monocytogenes [57]. The maintenance of CD8+ T cells and LIP require the integration of signals triggered on one hand by the TCR, upon weak stimulation delivered through self-pMHC interactions, and on the other hand by specific cytokine receptors involved in CD8+ T cell homeostasis [63]. Stimulation of THEMIS deficient CD8+ T cells expressing the OT-1 TCR with peptides of different affinities ranges showed no significant effect of THEMIS deficiency on T cell responses, such as proliferation, production of TNFα and apoptosis, and on TCR signaling events such the phosphorylation of ERK, suggesting modest regulatory function of THEMIS on TCR signals when those emanate exclusively from the TCR in the absence of co-modulatory signals [57]. In a subsequent study, authors show that the proliferation of OT1+ CD8+ T cells was also reduced in the absence of THEMIS, when these cells were transferred into β2m-deficient host lacking pMHC-I complexes, indicating potential stimulatory function of THEMIS on cytokine-driven homeostatic maintenance of peripheral CD8+ T cells [58]. Further analysis performed in that study show that THEMIS is required to stimulate the proliferation of naïve OT1+ CD8+ T cells cultured in vitro with IL-2 or IL-15, two cytokines which have important function in the maintenance of CD8+ T cells [64]. Interestingly, Themis cKO CD8+ T cells showed normal response to IL-7, another cytokine important for peripheral CD8+ T cell maintenance, indicating that THEMIS operates selectively on a subset of receptors from the common cytokine receptor γ-chain (γc) family [57]. The loss of THEMIS was associated with an impaired metabolic readjustment in response to these cytokines, characterized by a slower glycolytic rate and a moderately reduced serine biosynthesis [58]. Analysis of proximal signaling events triggered by IL-2 and IL-15 stimulation showed that THEMIS positively regulates the phosphorylation of the kinases JAK1 and JAK3 and of the transcription factor STAT5 [58]. Finally, these signaling defects as well the defects in CD8+ T cell homeostasis and proliferative responses could be alleviated when Ptpn6 and Themis gene were both disrupted in peripheral T cells in conditional knock-out mouse models [57,58], confirming the repressive effect of THEMIS on SHP-1-mediated signaling previously reported in thymocytes and indicating further that this mechanism could possibly be engage in other T-cell subsets through TCR-independent signaling processes.

THEMIS cooperates with VAV1 to promote optimal suppressive function in Treg cells

Following the initial characterization of THEMIS, one study on the Brown-Norway (BN) rat line identified a spontaneous frameshift mutation in the gene encoding Themis, leading to the complete loss of THEMIS expression, which was associated with the spontaneous development of an inflammatory bowel disease (IBD) [59]. The secretion of IL-17 and IL-10 by
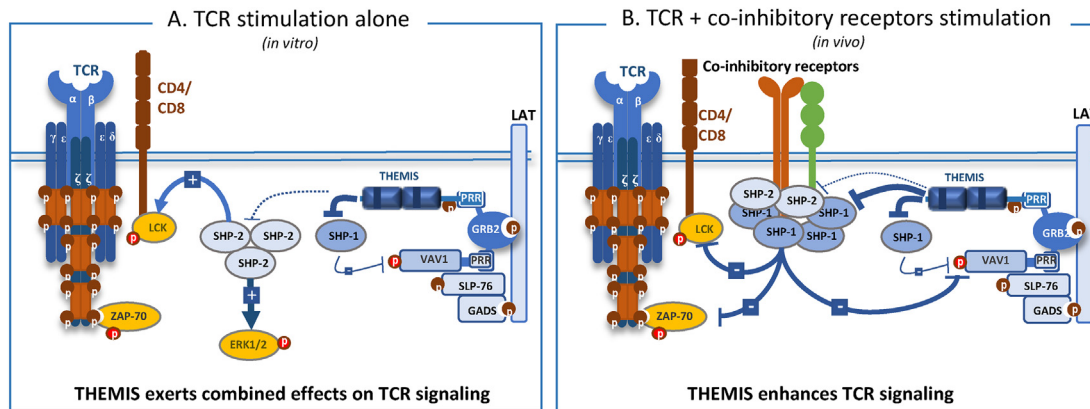
Fig. 3 The effect of THEMIS on TCR signaling is determined by the relative amounts of SHP-1 and SHP-2 that are recruited to TCR signaling complexes. A) THEMIS represses SHP-1 more strongly than SHP-2 yet, upon TCR stimulation alone (representative of TCR cross-linking *in vitro*), the positive effects of SHP-2 on TCR signaling predominates over SHP-1-mediated inhibitory effects. This might result in combined THEMIS-dependent outcomes on TCR signaling. B) The amount of SHP-1 that is recruited in proximity of TCR signaling complexes is increased following stimulation of co-inhibitory receptors. In the context of those receptors, SHP-2 operates as a negative regulator of TCR signaling. THEMIS might enhance TCR signaling by repressing the fraction of SHP-1 and SHP-2 recruited by those receptors.

THEMIS-deficient CD4+ T cells was exacerbated in response to TCR stimulation. Further analysis showed that THEMIS was required to promote optimal Treg suppressive function and that the prevalence of the disease could be reduced following transfer of wild-type Treg into the mutated rat lines [59]. In a following up study, authors showed that the same mutation transferred on the rat Lewis (LEW) background could neither impair Treg suppressive functions nor induce pathological manifestations, suggestive of an epistatic phenomenon occurring in the BN genetic background [52]. However, THEMIS-deficient CD4+ T cells from the LEW genetic background produced exacerbated amount of IL-17 and IL-10 in response to TCR stimulation [52], indicating that intrinsic defect associated to conventional CD4+ T cells could not alone explain the development of the disease. Because BN rats harbor a peculiar VAV1 variant (VAV1-W63) with reduced adaptor function and signaling capacities [65] and because THEMIS was shown to positively regulates VAV1 activity in thymocytes [14,43], authors hypothesized that the effect of THEMIS deficiency on BN Treg function could result from a cumulative impairment of the TCR signaling due to combined association of THEMIS deficiency with the presence of the VAV1-W63 variant. Accordingly, the introgression in the BN rat line of a 117-Kb interval bearing the LEW VAV1 variant could protect from IBD development and restore normal Treg suppressive function [59]. Although the precise influence of VAV1 in this epistasis phenomenon remains to be validated through gene-targeting approaches, this study suggests that the combination of genetic or epigenetic variants affecting concomitantly THEMIS and VAV1 expression could regulate the susceptibility to immune-based disorders. This might be physiologically relevant since VAV1 mRNA expression levels are highly variable in PBMCs from multiple sclerosis patients and healthy donors depending on VAV1 haplotypes [66]. The relatively low expression level of THEMIS in Treg cells as compared to conventional CD4+ T cells might contribute to

the reduced responsiveness of those cells to TCR stimulation [55,67]. Accordingly, the enforced expression of THEMIS in Treg cells through transgenic expression exacerbates their suppressive function in response to TCR engagement [55]. Thus, although THEMIS seems to stimulate Treg responses in VAV1 insufficient context, its expression in those cells is maintained at relatively low level possibly to reduce the responsiveness of those cells which are intrinsically highly self-reactive.

Does THEMIS regulate T cells through TCR-independent mechanism?

Although THEMIS interactome analysis by mass spectrometry indicates that THEMIS interacts mainly with proteins involved in TCR signaling [14], several observations suggest that THEMIS might not be operating exclusively downstream of the TCR and that additional stimulations might be required to promote THEMIS-dependent effect on TCR signaling *in vivo*. The loss of THEMIS has relatively little impact on TCR signaling in thymocytes and peripheral T cells when cells are stimulated promptly after purification with anti-CD3 antibodies [6–9,57]. The stimulation of peripheral CD8+ T cells with IL-2 or IL-15 also leads to reduced JAK-mediated signaling in the absence of THEMIS [58]. Finally, the discordancy between the effects of THEMIS on TCR signaling observed *in vitro* following TCR cross-linking with pMHC tetramers [35,36] and *in vivo* in the context of T cell development also support the possibility that THEMIS might operate independently of the TCR through additional receptors which may contribute to regulate TCR signal and selection processes. One striking observation is that THEMIS enhances the phosphorylation of ZAP-70 following thymocyte stimulation with concanavalin A, a reagent which engages multiple cell-surface molecules in addition to the TCR, but has no detectable effect on ZAP-70 phosphorylation following TCR cross-linking alone [13]. It was speculated that concanavalin A could elicit the

production of reactive oxygen species (ROS), which is poorly induced by TCR cross-linking in thymocytes [68], and which enables THEMIS to block SHP-1 phosphatase activity by promoting/stabilizing its cysteine active site in an oxidized state [13]. Accordingly, THEMIS was shown to enhance ZAP-70 and LCK phosphorylation following TCR cross-linking when cells were cultured in conditions that elicit ROS production [13]. However, the effects reported for THEMIS on LCK and ZAP-70 phosphorylation remained modest which calls for additional explanations to the defect of TCR signaling observed in THEMIS deficient thymocytes in response to concanavalin A [13]. It was proposed that SHP-1 operates downstream of the TCR in negative feedbacks loops which are engaged upon TCR recognition of weak affinity ligands [15]. Those feedback loops involve the kinase LCK which recruits phosphorylated SHP-1 through its SH2 domains upon weak agonists stimulation [15]. Upon recognition of strong agonist ligands by the TCR, LCK undergoes serine phosphorylation at position 59 that prevent the recruitment of SHP1 and subsequent TCR desensitization [15]. However, more recent analysis of knock-in mice in which the serine 59 was replaced by an alanine, revealed no detectable role of this feedback loop on T cell development and on the up-regulation of activation markers following TCR stimulation with agonists of different affinity ranges [36]. More generally, analysis based on SHP-1 deficient mice suggest that SHP-1 has a relatively mild impact on T cell responses *in vitro* upon TCR cross-linking [69–71]. This correlates with recent mass spectrometry results which fail to detect any robust interaction of SHP-1 with TCR signaling proteins following TCR stimulation with anti-CD3 antibodies [41]. However, the loss of SHP-1 leads to exacerbated T cell responses *in vivo* following immunization with self and foreign antigens [69,71–74]. This suggests that SHP-1 might function more effectively in feedback loops triggered by inhibitory receptors [17–19,75,76], which might enrich SHP-1 in proximity of TCR signaling complexes [Fig. 3]. Alternatively, SHP-1 might function in regulatory loops triggered by cytokine receptors, independently of the TCR [69,71]. A consequence of such mechanisms could be that although THEMIS inhibits SHP-1 predominantly over SHP-2, the functional consequence of inhibiting SHP-2 might predominate over that of inhibiting SHP-1 in the absence of co-inhibitory receptor stimulation [Fig. 3]. ITIM- and ITSM-containing receptors that recruit SHP-1 and SHP-2 are expressed during T cell development particularly at the stage of positive selection [77–79], suggesting that THEMIS might stimulate positive selection by blocking inhibitory signals transmitted by those receptors on TCR signaling. Of note, it was suggested recently that the bZIP factor of Human T-cell leukemia virus type 1 (HTLV-1) could interact with THEMIS and prevent the co-localization of THEMIS with PD-1 in T cells [80]. Mechanisms that repress PD-1 binding to its ligand PD-L1 [81] or that block TIM-3-mediated signaling [82] have been previously described, supporting the idea that those receptors are themselves submitted to a tight control of their activity. The possibility that THEMIS would operate as a natural repressor of ITIM-containing receptors could be of potential interest in the clinical field given the recent development of strategies based on the inhibition of these receptors in cancer immunotherapy.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgments

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