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### ► **To cite this version:**

Suzanne Mélique, Aurélie Vadel, Nelly Rouquié, Cui Yang, Cyrielle Bories, et al.. THEMIS promotes T cell development and maintenance by rising the signaling threshold of the inhibitory receptor BTLA. Proceedings of the National Academy of Sciences of the United States of America, 2024, 121 (20), pp.654-663. <10.1073/pnas.2318773121>. <hal-04757063>

**HAL Id: hal-04757063**

**<https://hal.science/hal-04757063v1>**

Submitted on 30 Oct 2024

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# THEMIS promotes T cell development and maintenance by rising the signaling threshold of the inhibitory receptor BTLA

Suzanne M<sup>é</sup>lique<sup>a</sup>, Aur<sup>é</sup>lie Vadel<sup>a</sup>, Nelly Rouqui<sup>é</sup><sup>a</sup> , Cui Yang<sup>a</sup> , Cyrielle Bories<sup>a</sup> , Coline Cotineau<sup>a</sup>, Abdelhadi Saoudi<sup>a</sup> , Nicolas Fazilleau<sup>a</sup> , and Renaud Lesourne<sup>a,1</sup> 

Edited by Kenneth Murphy, Washington University in St. Louis School of Medicine, St. Louis, MO; received October 26, 2023; accepted April 12, 2024

The current paradigm about the function of T cell immune checkpoints is that these receptors switch on inhibitory signals upon cognate ligand interaction. We here revisit this simple switch model and provide evidence that the T cell lineage protein THEMIS enhances the signaling threshold at which the immune checkpoint BTLA (B- and T-lymphocyte attenuator) represses T cell responses. THEMIS is recruited to the cytoplasmic domain of BTLA and blocks its signaling capacity by promoting/stabilizing the oxidation of the catalytic cysteine of the tyrosine phosphatase SHP-1. In contrast, THEMIS has no detectable effect on signaling pathways regulated by PD-1 (Programmed cell death protein 1), which depend mainly on the tyrosine phosphatase SHP-2. BTLA inhibitory signaling is tuned according to the THEMIS expression level, making CD8<sup>+</sup> T cells more resistant to BTLA-mediated inhibition than CD4<sup>+</sup> T cells. In the absence of THEMIS, the signaling capacity of BTLA is exacerbated, which results in the attenuation of signals driven by the T cell antigen receptor and by receptors for IL-2 and IL-15, consequently hampering thymocyte positive selection and peripheral CD8<sup>+</sup> T cell maintenance. By characterizing the pivotal role of THEMIS in restricting the transmission of BTLA signals, our study suggests that immune checkpoint operability is conditioned by intracellular signal attenuators.

T cell | immune checkpoint | signaling | BTLA | THEMIS

T cells express a set of inhibitory receptors (designated immune checkpoints) which enhance the threshold of T cell responsiveness to antigens and attenuate the magnitude of T cell effector responses by negatively regulating signals elicited by the T cell antigen receptor (TCR) and costimulatory receptors (1, 2). Therapies based on immune checkpoint blockade have revolutionized the field of cancer immunotherapy resulting in durable remission in patients with tumors that previously were uniformly fatal. However, a high proportion of patients are resistant to checkpoint blockade treatments (3–5), although targeted receptors are expressed on tumor-infiltrating immune cells, suggesting that additional layers of regulation modulate the functional capacity of these receptors.

B- and T-lymphocyte attenuator (BTLA) and Programmed cell death protein 1 (PD-1) are structurally related immune checkpoints that regulate T cell homeostasis and effector responses at multiple stages (6–8). Stimulation of BTLA and PD-1 by their natural ligands, Herpes virus entry mediator (HVEM) and Programmed death-ligand 1 (PD-L1) respectively, leads to the recruitment to their cytoplasmic tails of the protein tyrosine phosphatases (PTP) src homology-2 (SH2) domain-containing phosphatase (SHP)-1 and SHP-2 that dephosphorylate and inactivate signaling proteins, downstream of the TCR and CD28 (9–12). Each receptor recruits both phosphatases with different stoichiometries, with BTLA interacting predominantly with SHP-1 and PD-1 interacting predominantly with SHP-2 (10, 11). The T-lineage protein thymocyte-expressed molecule involved in Selection (THEMIS) has been identified as an inhibitor of the catalytic activity of SHP-1 and to a lesser extent of SHP-2 (13). This action is mediated by globular domains on THEMIS, named cysteine-containing, all-beta in Themis (CABIT) modules (14), which bind to the PTP domain of SHP1/2 and promote oxidation of their catalytic cysteine by reactive oxygen species, resulting in inhibition of PTP activity (13, 15). Analysis of THEMIS-deficient mouse models revealed that THEMIS is critical for T cell development (14, 16, 17) and peripheral CD8<sup>+</sup> T cell homeostasis (18). Although the *in vivo* defects associated with THEMIS deficiency are suggestive of reduced TCR signal intensity, most studies have failed to detect significant signaling defects caused by the loss of THEMIS following TCR cross-linking *in vitro* (14, 16, 17, 19). This indicates that THEMIS could operate in the context of other signaling receptors.

Here, we found that THEMIS enhances TCR signals by restraining the signaling capacity of the inhibitory receptor BTLA. THEMIS is recruited to the cytoplasmic tail of BTLA together with the adapter protein GRB2 (Growth factor receptor-bound protein) and inactivates SHP-1 by promoting/stabilizing the oxidation of its catalytic cysteine. In

## Significance

Therapies based on immune checkpoint blockades have revolutionized the field of cancer immunotherapy, yet the molecular mechanism underlying the signaling activity of these receptors remains ill-defined. Here, we show that the T cell lineage protein THEMIS operates as a rheostat which restrain the signaling capacity the inhibitory receptor BTLA (B- and T-lymphocyte attenuator). THEMIS promotes T cell development and maintenance by conferring to T cells the capacity to resist to BTLA-mediated inhibition. Our findings suggest that the operability of immune checkpoints depends on intracellular factors that regulate the signaling thresholds at which these receptors inhibit T cell responses. This improves our understanding of immune checkpoint operability which was mainly described as a simple switch mechanism relying solely on cognate ligand interactions.

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Author contributions: S.M., A.S., N.F., and R.L. designed research; S.M., A.V., N.R., C.Y., C.B., and C.C. performed research; S.M., C.B., C.C., N.F., and R.L. analyzed data; and R.L. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at <https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2318773121/-/DCSupplemental>.

Published May 7, 2024.

contrast, THEMIS has no detectable regulatory effect on the PD-1-SHP-2 inhibitory axis. THEMIS operates as a rheostat, conferring on T cells the capacity to resist to BTLA-mediated inhibition according to its expression levels, which is variable across T cell subsets. This mechanism is critical for thymocyte development, enabling signals elicited by the TCR to reach the threshold of positive selection, and for the maintenance of naive CD8+ T cells, enhancing homeostatic signals driven by low-affinity TCR ligands and cytokines.

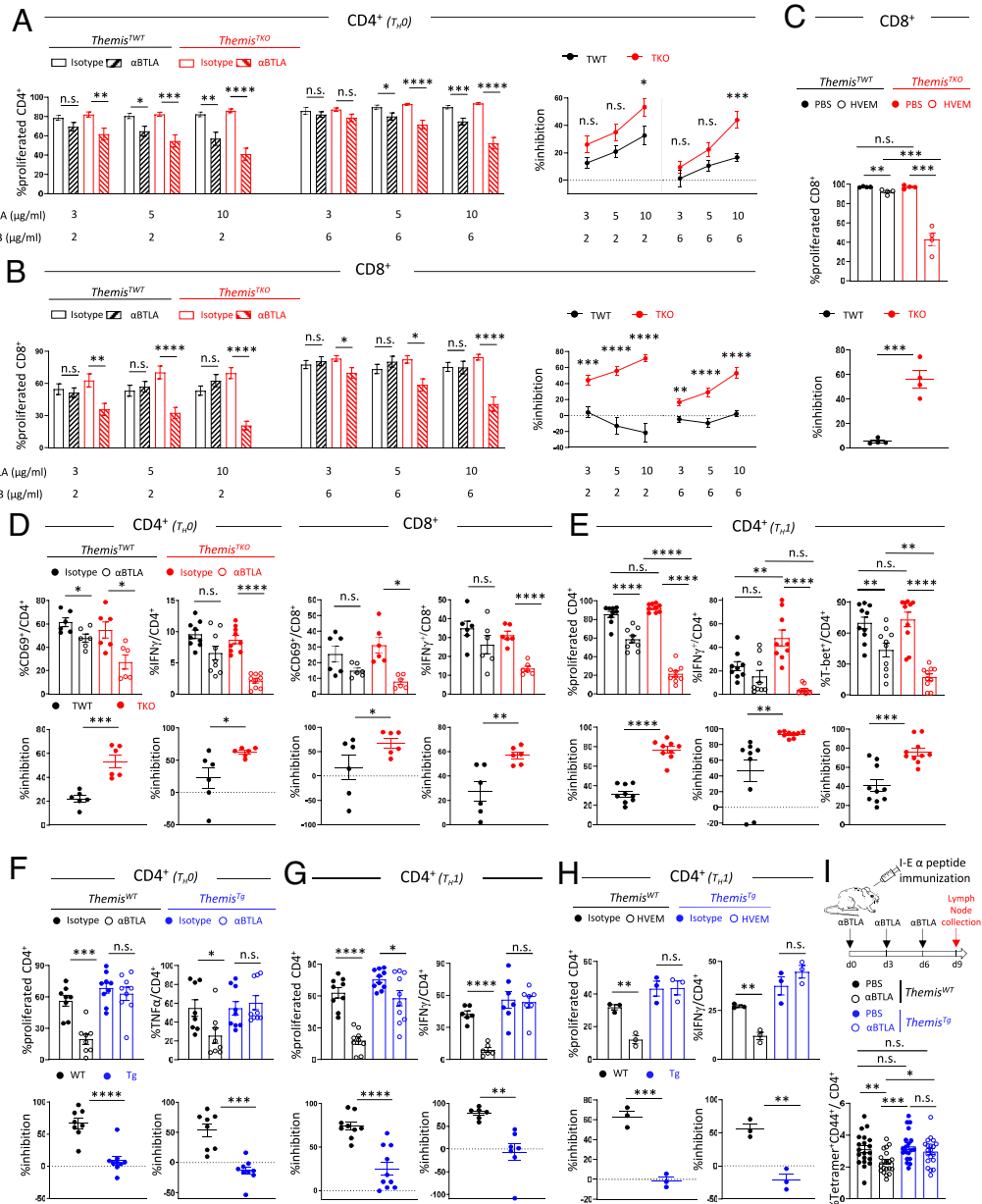
## Results

**The Inhibitory Function of BTLA Is Repressed by THEMIS in Peripheral T cells.** We hypothesized that THEMIS could regulate the inhibitory functions of BTLA and PD-1 and first focused our investigations on BTLA which interacts predominantly with SHP-1 (11, 20), a major binding partner of THEMIS (21). The ability of THEMIS to modulate the inhibitory functions of BTLA was analyzed in vitro in T cells isolated from mice that harbor a conditional deletion of the *Themis* gene in peripheral T cells (*Cd2-Cre+ Themis<sup>flx/flx</sup>*, referred to as *Themis<sup>TKO</sup>* mice) (22). We first stimulated naive CD4+ and CD8+ T cells from *Cd2-Cre-neg Themis<sup>flx/flx</sup>* (referred to as *Themis<sup>TWT</sup>*) and *Themis<sup>TKO</sup>* mice with variable amounts of plate-bound anti-CD3 antibodies in the presence of different concentrations of agonist antibodies against BTLA or isotype control antibody and compared the ability of BTLA to inhibit the proliferation of cells induced in response to TCR stimulation. In the presence of THEMIS, the costimulation of the TCR and BTLA mildly inhibited the proliferation of CD4+ T cells but had no detectable inhibitory effects on the percentages of proliferating CD8+ T cells (Fig. 1 *A* and *B* and *SI Appendix, Fig. S1*). The inhibitory effects of BTLA in CD4+ T cells increased with the amount of stimulatory antibody against BTLA, resulting in a 25% decrease of proliferation at the highest antibody doses (Fig. 1*A* and *SI Appendix, Fig. S1*). In the absence of THEMIS in similar conditions, the stimulation of BTLA together with the TCR decreased by 50% the percentage of cells that proliferated, indicating increased inhibitory capacity of BTLA (Fig. 1*A* and *SI Appendix, Fig. S1*). Most strikingly, the absence of THEMIS conferred on BTLA the capacity to inhibit CD8+ T cell proliferation, reaching 60% inhibition when cells were stimulated with a combination of low and high amounts of anti-TCR and anti-BTLA antibodies, respectively (Fig. 1*B* and *SI Appendix, Fig. S1*). Similar increase of BTLA inhibitory functions were observed in THEMIS-deficient CD8+ T cells when BTLA was stimulated with recombinant HVEM, indicating that this effect was conserved when BTLA was stimulated with its natural ligand (Fig. 1*C*). The loss of THEMIS had no detectable effect on BTLA surface expression upon antibody-mediated TCR and CD28 stimulation (*SI Appendix, Fig. S2A*), indicating that the increased ability of BTLA to inhibit T cell activation in the absence of THEMIS was not the consequence of higher BTLA surface expression.

The capacity of BTLA to inhibit the expression of activation markers and the production of cytokines induced upon TCR stimulation was also increased in *Themis<sup>TKO</sup>* CD4+ and CD8+ T cells in comparison to that in control *Themis<sup>TWT</sup>* cells (Fig. 1*D*). We previously showed that the loss of THEMIS leads to an exacerbated production of IFN $\gamma$  by CD4+ T cells stimulated in T<sub>H</sub>1 polarizing conditions in the presence of anti-CD3 antibodies, whereas in vivo, THEMIS deficiency results in a decreased production of IFN $\gamma$  and expression of the transcription factor T-box Expressed in T cells (T-BET) in effector CD4+ T cells specific of self- or foreign antigens (22). We hypothesized that this

discrepancy could reflect the ability of THEMIS to modulate the signaling capacity of receptors which would not be engaged in a reductionist in vitro model exclusively based on TCR stimulation (22, 23). Thus, we examined whether THEMIS could stimulate the production of IFN $\gamma$  and T-BET in T<sub>H</sub>1-polarized cells by repressing the ability of BTLA to inhibit those responses. In agreement with our previous observation (22), the loss of THEMIS was associated with an enhanced production of IFN $\gamma$  in CD4+ T cells stimulated in T<sub>H</sub>1 conditions with no detectable effect on T-BET expression (Fig. 1*E* and *SI Appendix, Fig. S1*). The proliferation and the production of IFN $\gamma$ , as well as the expression of the transcription factor T-BET, were mildly inhibited by BTLA in THEMIS-sufficient CD4+ T cells polarized in T<sub>H</sub>1 conditions but became strongly repressed upon BTLA stimulation in the absence of THEMIS (Fig. 1*E*), recapitulating in vitro the stimulatory effect of THEMIS on T<sub>H</sub>1 responses that was previously detected in vivo (22). Together, these results indicated that the loss of THEMIS increased the capacity of BTLA to inhibit the activation of CD4+ and CD8+ T cells and the differentiation of naive CD4+ T cells into T<sub>H</sub>1 cells.

We noticed that the capacity of BTLA to inhibit T cell activation was more pronounced in CD4+ T cells than in CD8+ T cells (Fig. 1 *A* and *B*). We hypothesized that this could result from the lower amount of THEMIS expressed in CD4+ T cells relatively to that in CD8+ T cells (*SI Appendix, Fig. S2B* and ref. 24). We thus analyzed whether enhancing the amount of THEMIS in CD4+ T cells, through transgenic expression (*Themis<sup>Tg</sup>* mice), could repress the inhibitory functions of BTLA. The expression level of THEMIS in CD4+ T cells from *Themis<sup>Tg</sup>* mice was increased by eightfold relatively to that in wild-type cells, far beyond the amount of THEMIS expressed in CD8+ T cells (*SI Appendix, Fig. S2B*). We then stimulated naive CD4+ T cells from *Themis<sup>WT</sup>* and *Themis<sup>Tg</sup>* mice with a low amount of plate-bound anti-CD3 antibodies, which favored inhibition of CD4+ T cell responses upon BTLA stimulation (Fig. 1 *A* and *F*). The ability of BTLA to inhibit CD4+ T cell proliferation was blocked in the context of THEMIS transgenic expression (Fig. 1*F*). Confirming the stimulatory effect of THEMIS on T<sub>H</sub>1 responses, we also observed that the transgenic expression of THEMIS could block BTLA-mediated inhibition in T<sub>H</sub>1-polarized cells and restored proliferation and production of IFN $\gamma$  to levels comparable to those observed when the TCR was stimulated alone (Fig. 1*G*). THEMIS transgenic expression could also block BTLA-mediated inhibition of T<sub>H</sub>1 responses when cells were stimulated with recombinant HVEM (Fig. 1*H*). To determine whether THEMIS transgenic expression could repress the inhibitory effect of BTLA on CD4+ T cell expansion in vivo, we administered BTLA agonist antibodies to *Themis<sup>WT</sup>* and *Themis<sup>Tg</sup>* mice previously immunized with a peptide variant of the I-E  $\alpha$  chain immunodominant peptide 52-68 (EAWGALANKAVDKA, hereafter called the I-E  $\alpha$  peptide), and followed antigen-specific CD4+ T cells by staining cells from the draining lymph nodes with the I-E $\alpha$ -pMHCII tetramer. In *Themis<sup>WT</sup>* mice, the percentages of tetramer+ CD4+ T cells were decreased upon injection of BTLA agonist antibodies in comparison to that in the control PBS group, reflecting a decreased expansion of antigen-specific CD4+ T cells (Fig. 1*I*). In contrast, injection of BTLA agonist antibodies in *Themis<sup>Tg</sup>* mice had no detectable effects on the percentages of tetramer+ CD4+ T cells (Fig. 1*I*), indicating that THEMIS transgenic expression restrained the capacity of BTLA to inhibit the expansion of antigen-specific CD4+ T cells. Collectively, these results demonstrated that THEMIS enhances peripheral T cell responses by repressing the inhibitory function of BTLA in peripheral T cells.

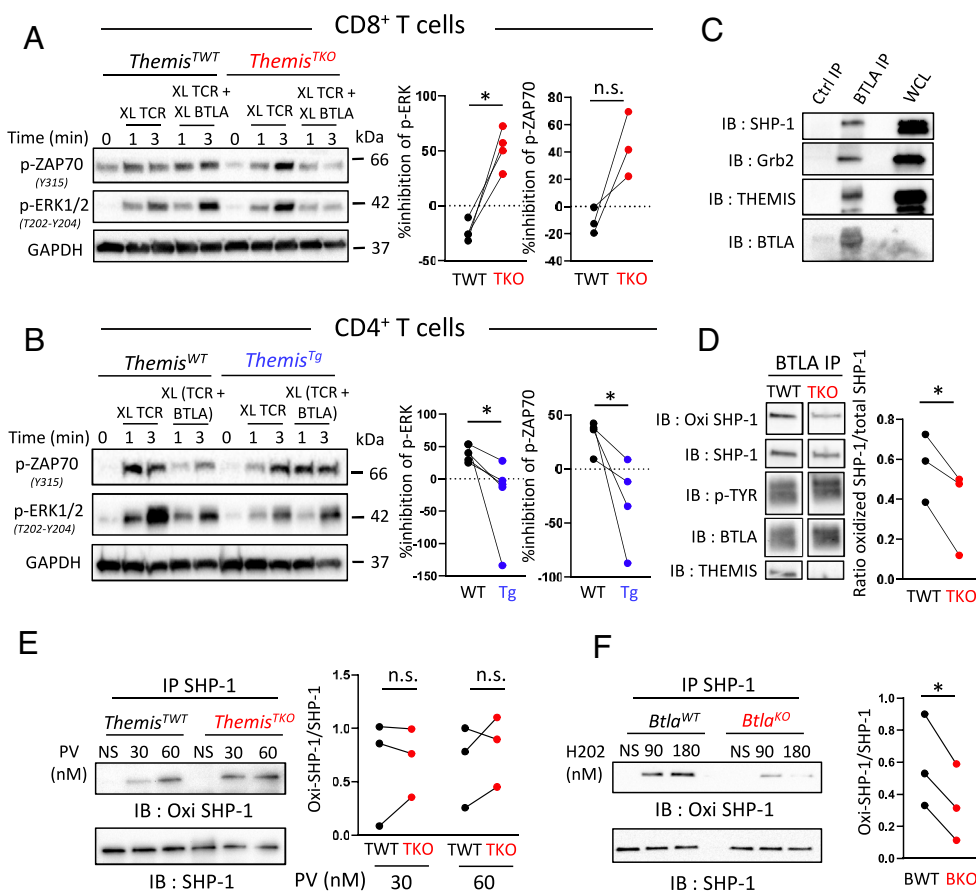


**Fig. 1.** THEMIS represses the inhibitory functions of BTLA in peripheral T cells. (A and B) Naive CD44-CD25- CD4<sup>+</sup> T cells (A) and CD8<sup>+</sup> T cells (B) from *Themis*<sup>TWT</sup> and *Themis*<sup>TKO</sup> mice were stained with CellTrace violet (CTV) and stimulated with the indicated doses of anti-CD3 and anti-CD28 antibodies in the presence of the indicated doses of plate-bound anti-BTLA antibodies or isotype control antibody for 72 h. Bar graphs represent the percentages of cells that divided at least one time as determined by flow cytometry at 72 h. Curves represent the percentages of inhibition of proliferation by BTLA in CD4<sup>+</sup> and CD8<sup>+</sup> T cells from *Themis*<sup>TWT</sup> (black curves) and *Themis*<sup>TKO</sup> (red curves) mice. Data are mean ± SEM and represent four to five independent experiments each including n = 3 mice per group. (C) Naive CD44-CD25- CD8<sup>+</sup> T cells from *Themis*<sup>TWT</sup> and *Themis*<sup>TKO</sup> mice were stimulated with anti-CD3 and anti-CD28 antibodies in the presence or not of plate-bound HVEM-Fc recombinant protein. Bar graphs represent the percentages of cells that proliferated. Dot graphs represent the percentage of inhibition of proliferation by BTLA in CD8<sup>+</sup> T cells from *Themis*<sup>TWT</sup> (black curves) and *Themis*<sup>TKO</sup> (red curves) mice. (D) Naive CD44-CD25- CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells from *Themis*<sup>TWT</sup> and *Themis*<sup>TKO</sup> mice were stimulated with anti-CD3 (6 to 8 μg/ml) and anti-CD28 antibodies in the presence of plate-bound anti-BTLA antibodies or isotype control antibody. Bar graphs represent the percentages of cells that are positive for CD69 and IFN $\gamma$  staining as determined by flow cytometry at 24 and 72 h respectively. Dot graphs represent the percentages of inhibition of each response by BTLA in CD4<sup>+</sup> and CD8<sup>+</sup> T cells from *Themis*<sup>TWT</sup> (black dots) and *Themis*<sup>TKO</sup> (red dots) mice. Data are mean ± SEM and represent two to three independent experiments each including n = 3 mice per group. (E) Naive CD44-CD25- CD4<sup>+</sup> T cells from *Themis*<sup>TWT</sup> and *Themis*<sup>TKO</sup> mice were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of plate-bound anti-BTLA antibodies or isotype control in T<sub>H1</sub> polarizing cell-culture medium for 72 h. Bar graphs represent the percentages of cells that proliferated and that are positive for IFN $\gamma$  and T-BET as determined by flow cytometry. Dot graphs represent the percentages of inhibition of each response by BTLA in CD4<sup>+</sup> T cells from *Themis*<sup>TWT</sup> (black dots) and *Themis*<sup>TKO</sup> (red dots) mice. Data are mean ± SEM and represent three independent experiments each including n = 3 to 4 mice per group. (F and G) Naive CD44-CD25- CD4<sup>+</sup> T cells from *Themis*<sup>WT</sup> and *Themis*<sup>Tg</sup> mice were stained with CTV and stimulated with anti-CD3 (2 μg/ml) and anti-CD28 antibodies in the presence of plate-bound anti-BTLA antibodies or isotype control antibody in T<sub>H0</sub> (F) or T<sub>H1</sub> (G) polarizing cell-culture medium. Bar graphs represent the percentages of cells that proliferated and produced TNF $\alpha$  or IFN $\gamma$  as determined by flow cytometry at 72 h. Data are mean ± SEM and represent two to three independent experiments each including n = 3 mice per group. (H) Naive CD44-CD25- CD4<sup>+</sup> T cells from *Themis*<sup>WT</sup> and *Themis*<sup>Tg</sup> mice were stimulated as in (G) in the presence of recombinant HVEM instead of anti-BTLA agonist antibodies. Bar graphs represent the percentages of cells that proliferated and produced IFN $\gamma$  as determined by flow cytometry at 72 h. Dot graphs represent the percentages of inhibition of each response by BTLA in CD4<sup>+</sup> T cells from *Themis*<sup>WT</sup> (black dots) and *Themis*<sup>Tg</sup> (blue dots) mice. Data are mean ± SEM and represent the experiment including n = 3 mice per group. (I) *Themis*<sup>WT</sup> and *Themis*<sup>Tg</sup> mice were immunized subcutaneously with I-E $\alpha$  peptide in CFA. Anti-BTLA antibodies or PBS were injected intravenously (200 μg/mouse) at the day of immunization and at days 3 and 6 postimmunization. Draining lymph nodes were collected after 9 d, and the isolated cells were stained with I-E $\alpha$ -conjugated MHC tetramers. Bar graphs represent the percentages of tetramer+CD44+CD4<sup>+</sup> T cells among CD4<sup>+</sup> T cells as determined by flow cytometry. Data are mean ± SEM and represent four independent experiments each including n = 4 to 5 mice per group. Data were analyzed by the unpaired two-tailed Mann-Whitney *t* test. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, and \*\*\*\**P* < 0.0001.

**THEMIS Enhances TCR Signaling by Regulating the Oxidation of SHP-1 Bound to BTLA.** We next investigated whether THEMIS could restrict the capacity of BTLA to regulate signals triggered by the TCR. To address this question, we stimulated CD4<sup>+</sup> and CD8<sup>+</sup> T cells from *Themis*<sup>TWT</sup>, *Themis*<sup>TKO</sup>, and *Themis*<sup>Tg</sup> mice with anti-CD3 and anti-CD28 antibodies for 2 d, to induce BTLA surface expression, and rested the cells in interleukine (IL)-2-containing medium, prior stimulation with anti-CD3 antibodies in the presence or not of anti-BTLA antibodies. We first analyzed whether the absence of THEMIS would enable BTLA to repress TCR signals in CD8<sup>+</sup> T cells since they were more refractory to BTLA-mediated inhibition than CD4<sup>+</sup> T cells. The phosphorylation of Zeta-chain-associated protein kinase 70 (ZAP-70) and Extracellular signal-regulated kinases (ERK) -1 and -2 were induced upon TCR cross-linking but were not detectably attenuated by BTLA costimulation in *Themis*<sup>TWT</sup> CD8<sup>+</sup> T cells (Fig. 2A). In contrast, ZAP-70 and ERK1/2 phosphorylation became susceptible to BTLA-mediated inhibition when cells

were stimulated in similar conditions in the absence of THEMIS (Fig. 2A). We next evaluated whether the transgenic expression of THEMIS could repress BTLA-dependent inhibitory signals in CD4<sup>+</sup> T cells, which were more amenable than CD8<sup>+</sup> T cells to BTLA-mediated inhibition. Confirming previous reports (21), the amount of phosphorylated ERK induced after TCR cross-linking was lower in *Themis*<sup>Tg</sup> CD4<sup>+</sup> T cells compared to that in *Themis*<sup>TWT</sup> cells (Fig. 2B), possibly because THEMIS mildly inhibits SHP-2 PTP's activity, which is required for ERK1/2 activation (25, 26). The phosphorylation of ZAP-70 and ERK1/2 was decreased upon BTLA stimulation in wild-type CD4<sup>+</sup> T cells but became resistant to BTLA-mediated inhibition in CD4<sup>+</sup> T cells expressing THEMIS transgene (Fig. 2B). Together, these results indicated that THEMIS promoted TCR signaling by repressing BTLA-mediated negative regulation.

We next examined the mechanism by which THEMIS represses BTLA-mediated signaling. BTLA stimulation by its ligand leads to the recruitment of the adapter protein GRB2 to its cytoplasmic



**Fig. 2.** THEMIS stimulates TCR signaling by enhancing the oxidation of the SHP-1 catalytic cysteine on BTLA. (A and B) The TCR and BTLA were cross-linked on pre-expanded CD8<sup>+</sup> T cells from *Themis*<sup>TWT</sup> and *Themis*<sup>TKO</sup> mice (A) and on pre-expanded CD4<sup>+</sup> T cells from *Themis*<sup>TWT</sup> and *Themis*<sup>Tg</sup> mice (B) for the indicated times. Total cytoplasmic extracts of the cells were then analyzed by western blotting with antibodies against phosphorylated forms of ZAP-70 (Tyr315) and ERK1/2 (Thr202/Tyr204). Graphs represent the percentages of inhibition by BTLA of ERK and ZAP-70 phosphorylation after 3 min of stimulation. The percentages of inhibition were calculated in each experiment from the relative abundances of the indicated phosphorylated proteins to those corresponding to GAPDH, the loading control. Paired black and red dots represent 3 to 4 independent experiments. (C) Pre-expanded CD8<sup>+</sup> T cells were stimulated with pervanadate for 5 min. Samples were then subjected to immunoprecipitation (IP) with antibodies specific for BTLA or isotype control and then analyzed by western blotting with antibodies specific for the indicated proteins. WCL, whole cell lysate. (D) Pre-expanded CD8<sup>+</sup> T cells from *Themis*<sup>TWT</sup> and *Themis*<sup>TKO</sup> mice were stimulated with pervanadate for 5 min. Samples were then subjected to IP with antibodies specific for BTLA and then analyzed by western blotting with antibodies specific for the indicated proteins. The graph represents the ratios of the intensities of the bands corresponding to the oxidized form of SHP-1 to those corresponding to SHP-1. Paired dots represent three independent experiments. (E) Pre-expanded CD8<sup>+</sup> T cells from *Themis*<sup>TWT</sup> and *Themis*<sup>TKO</sup> mice were stimulated with pervanadate at the indicated doses for 5 min. Samples were then subjected to IP with antibodies specific for SHP-1 and then analyzed by western blotting with antibodies specific for the indicated proteins. The graph represents the ratios of the intensities of the bands corresponding to the oxidized form of SHP-1 to those corresponding to SHP-1. Paired dots represent three independent experiments. (F) Pre-expanded CD8<sup>+</sup> T cells from *Btla*<sup>WT</sup> (BWT) and *Btla*<sup>KO</sup> (BKO) mice were stimulated with H<sub>2</sub>O<sub>2</sub> for 5 min. Samples were then subjected to IP with antibodies specific for SHP-1 and then analyzed by western blotting with antibodies specific for the indicated proteins. The graph represents the ratios of the intensities of the bands corresponding to the oxidized form of SHP-1 to those corresponding to SHP-1. Paired dots represent three independent experiments. Data were analyzed by paired two-tailed Mann-Whitney *t* test. \**P* < 0.05.

tail, which contains a tyrosine-based YxN sequence typical of GRB2-binding motif (27). Quantitative mass-spectrometry studies indicate that GRB2 is the major binding partner of THEMIS in thymocytes (21) and peripheral T cells (28), suggesting that THEMIS could interact with BTLA through GRB2. Accordingly, THEMIS, GRB2 and SHP-1 were precipitated together with BTLA in expanded CD8+ T cells stimulated with pervanadate, a pan-tyrosine phosphatase inhibitor (Fig. 2C). The amount of SHP-1 that precipitated together with BTLA was not significantly modified in *Themis*<sup>TKO</sup> CD8+ T cells, indicating that THEMIS does not operate by repressing SHP-1 interaction with BTLA (Fig. 2D). Recent studies demonstrated that GRB2 facilitates THEMIS-mediated inactivation of SHP-1 (29), leading us to hypothesize that the SHP-1 recruited to BTLA could be inactivated by THEMIS. To address this possibility, we analyzed the amount of oxidized (inactivated) SHP-1 bound to BTLA upon treatment of expanded CD8+ T cells with pervanadate, which irreversibly oxidizes PTP active-site cysteine residues to the sulfonic acid (S-O<sub>3</sub>H) form (30). Oxidized SHP-1 was detected by immunoblot analysis with a monoclonal antibody specific for sulfonated PTP active-site cysteines (31). The amount of oxidized SHP-1 that precipitated together with BTLA was enhanced in the presence of THEMIS (Fig. 2D). In contrast, the amount of total cellular oxidized SHP-1 was not significantly increased in *Themis*<sup>TWT</sup> CD8+ T cells compared to *Themis*<sup>TKO</sup> CD8+ T cells (Fig. 2E), indicating that THEMIS regulates SHP-1's catalytic cysteine when both proteins assembled together on BTLA cytoplasmic tail. Supporting this possibility, the amount of oxidized SHP-1 was decreased in *Btla*<sup>KO</sup> CD8+ T cells as compared to that in wild-type cells following treatment with H<sub>2</sub>O<sub>2</sub>, which induces SHP-1 oxidation (Fig. 2F). Together, these results indicated that THEMIS inactivates SHP-1 upon its recruitment to BTLA.

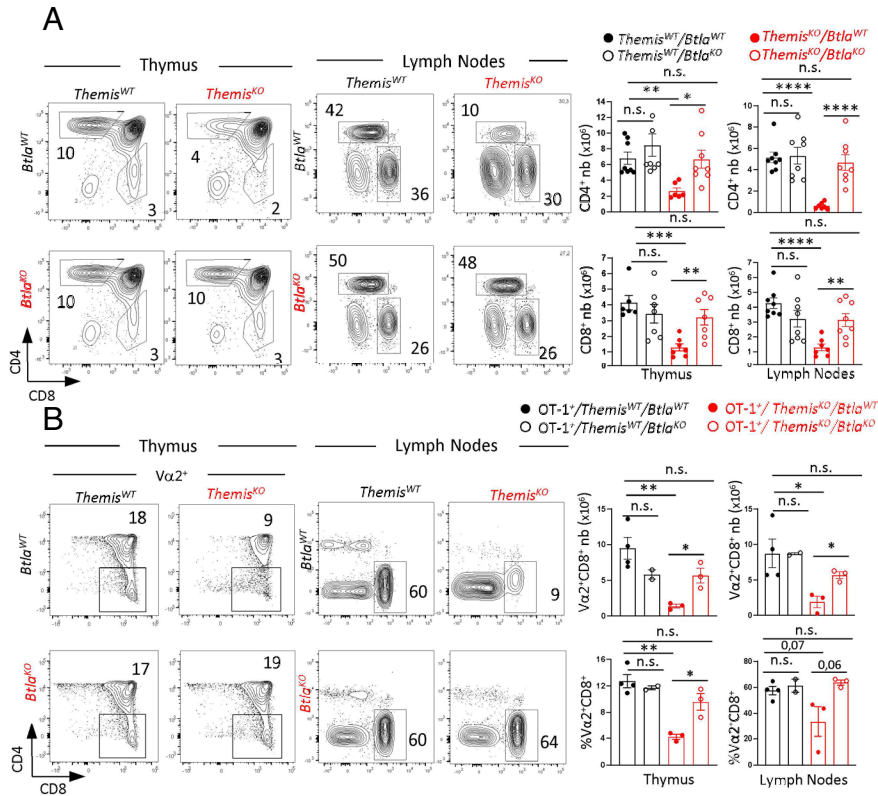
**Restriction of BTLA Inhibitory Function by THEMIS Enhances Thymocyte Positive Selection.** BTLA expression is induced during T cell development at the stage of thymocyte positive selection (32), yet mice deficient for BTLA do not show significant defects of T cell development (9), suggesting that BTLA might be partially inoperative in this context. Of note, mice bearing a germline deletion in the *Themis* gene (*Themis*<sup>KO</sup>) exhibit a severe defect of thymocyte positive selection, characterized by partial block of T cell development at the transition stage from CD4+CD8+ thymocytes [referred to as double-positive (DP) thymocytes] to CD4+CD8- and CD4-CD8+ thymocytes [referred to as single-positive (SP) thymocytes]. This defect is associated with an attenuation of TCR signaling (16, 21) and could be alleviated by deleting the gene encoding for SHP-1 (13, 33), suggesting that attenuation of BTLA-mediated signaling by THEMIS could be required for enabling TCR signals to reach the threshold of positive selection.

To address this possibility, we first analyzed BTLA surface expression on thymocyte subsets from mice expressing or not the  $\alpha\beta$ -TCR transgene (OT-1) restricted to the class-I MHC. BTLA was not detected on preselection DP CD69- thymocytes but was expressed on DP CD69+, at early stages of positive selection, and increased further in expression on postselection CD69+CD4+ and CD8+ SP thymocytes (SI Appendix, Fig. S3A), confirming previous observations (32). HVEM was also expressed on cortical thymic epithelial cells (TECs) and to a lesser extent on medullary TECs, which are involved in self-antigen presentation to thymocytes in the context of positive and negative selection, respectively (SI Appendix, Fig. S3B). To determine whether the developmental block in *Themis*<sup>KO</sup> mice could result from increased BTLA-mediated inhibition of TCR signaling, we crossed mice bearing a germline

deletion of *Themis* (*Themis*<sup>KO</sup>) with *Btla*<sup>KO</sup> mice to generate mice deficient for both THEMIS and BTLA (*Themis*<sup>KO</sup>*Btla*<sup>KO</sup>). Thymocytes from *Themis*<sup>KO</sup>*Btla*<sup>TWT</sup> control mice exhibited normal maturation up to the DP stage but a marked reduction in the numbers of CD4+ and, to a lesser extent, of CD8+ SP thymocytes and peripheral T cells (Fig. 3A). The deletion of *Btla* rescued the developmental block, as evinced by the significantly greater frequency and number of CD4+ and CD8+ SP thymocytes and peripheral T cells in *Themis*<sup>KO</sup>*Btla*<sup>KO</sup> mice which reached the values observed in control *Themis*<sup>TWT</sup>*Btla*<sup>TWT</sup> mice (Fig. 3A). The percentages of TCR<sup>hi</sup> and the numbers of mature CD24<sup>low</sup> SP thymocytes, which are decreased in *Themis*<sup>KO</sup>*Btla*<sup>TWT</sup> mice, were also restored in *Themis*<sup>KO</sup>*Btla*<sup>KO</sup> mice to values comparable to those observed in *Themis*<sup>TWT</sup>*Btla*<sup>TWT</sup> mice (SI Appendix, Fig. S4A and B). As reported previously (16), the surface expression of CD5, which is correlated with the strength of TCR signals (34), was decreased on immature TCR+CD62L<sup>low</sup> CD4+ and CD8+ SP thymocytes from *Themis*<sup>KO</sup>*Btla*<sup>TWT</sup> mice (SI Appendix, Fig. S4C). The deletion of *Btla* in *Themis*<sup>KO</sup> mice restored CD5 surface expression on these cells to levels comparable to those observed in *Themis*<sup>TWT</sup>*Btla*<sup>TWT</sup> mice (SI Appendix, Fig. S4C), indicating that the defect of TCR signaling in the THEMIS-deficient thymocytes is caused by BTLA-dependent inhibitory signals. T cell development was also analyzed in *Themis*<sup>KO</sup>*Btla*<sup>KO</sup> mice expressing the  $\alpha\beta$ -TCR transgene OT-1, which drives positive selection signals leading to CD8+ SP thymocyte development (35). The numbers and percentages of V $\alpha$ 2+CD8+ SP thymocytes, which represent CD8+ thymocytes expressing the OT-1 TCR, were strongly reduced in *Themis*<sup>KO</sup>*Btla*<sup>TWT</sup> mice, similar to what has been described previously (17), but were increased in *Themis*<sup>KO</sup>*Btla*<sup>KO</sup> mice to similar values as that observed in *Themis*<sup>TWT</sup>*Btla*<sup>TWT</sup> mice (Fig. 3B). Collectively, these results demonstrate that the attenuation of BTLA-mediated signaling by THEMIS is required for optimal T cell development.

**PD-1 Operates Independently of THEMIS in Thymocytes and Peripheral T cells.** We then evaluated whether the inhibitory potential of PD-1, which relies predominantly on SHP-2 (10), could also be attenuated by THEMIS. The deletion of *Themis* had no detectable effect on PD-1 surface level after TCR stimulation (SI Appendix, Fig. S2A). Naive CD8+ T cells from *Themis*<sup>TWT</sup>, *Themis*<sup>TKO</sup>, and *Themis*<sup>Tg</sup> mice were stimulated with anti-CD3 antibodies in the presence or not of different amounts of PD-L1-Fc recombinant proteins or isotype control antibody. Stimulation of PD-1 moderately inhibited the proliferation of CD8+ T and only at the highest dose of plate-bound PD-L1 (Fig. 4A). In contrast to what we previously observed upon BTLA engagement, the deletion of THEMIS did not enhance the ability of PD-1 to inhibit CD8+ T cell proliferation (Fig. 4A). Stimulation of PD-1 also inhibited similarly the production of cytokines in *Themis*<sup>TWT</sup> and *Themis*<sup>TKO</sup> CD8+ T cells (SI Appendix, Fig. S5A). The transgenic expression of THEMIS, which increases the abundance of THEMIS by about fourfold in CD8+ T cells (SI Appendix, Fig. S2B), also did not significantly attenuate the inhibitory effects of PD-1 on proliferation and cytokine secretion (Fig. 4B and SI Appendix, Fig. S5B). Taken together, these results indicated that modulating THEMIS expression does not modify the capacity of PD-1 to inhibit peripheral T cell responses.

Since PD-1 was reported to negatively regulate thymocyte positive selection (36), we also analyzed T cell development in mice deficient for both THEMIS and PD-1 (*Themis*<sup>KO</sup>*Pdcd1*<sup>KO</sup>). In contrast to what we observed in *Themis*<sup>KO</sup>*Btla*<sup>KO</sup> mice, the deletion of *Pdcd1* in *Themis*<sup>KO</sup> mice did not increase the percentages and numbers of CD4+ SP thymocytes and peripheral T cells (Fig. 4C). The percentages and numbers of CD8+ SP thymocytes, including

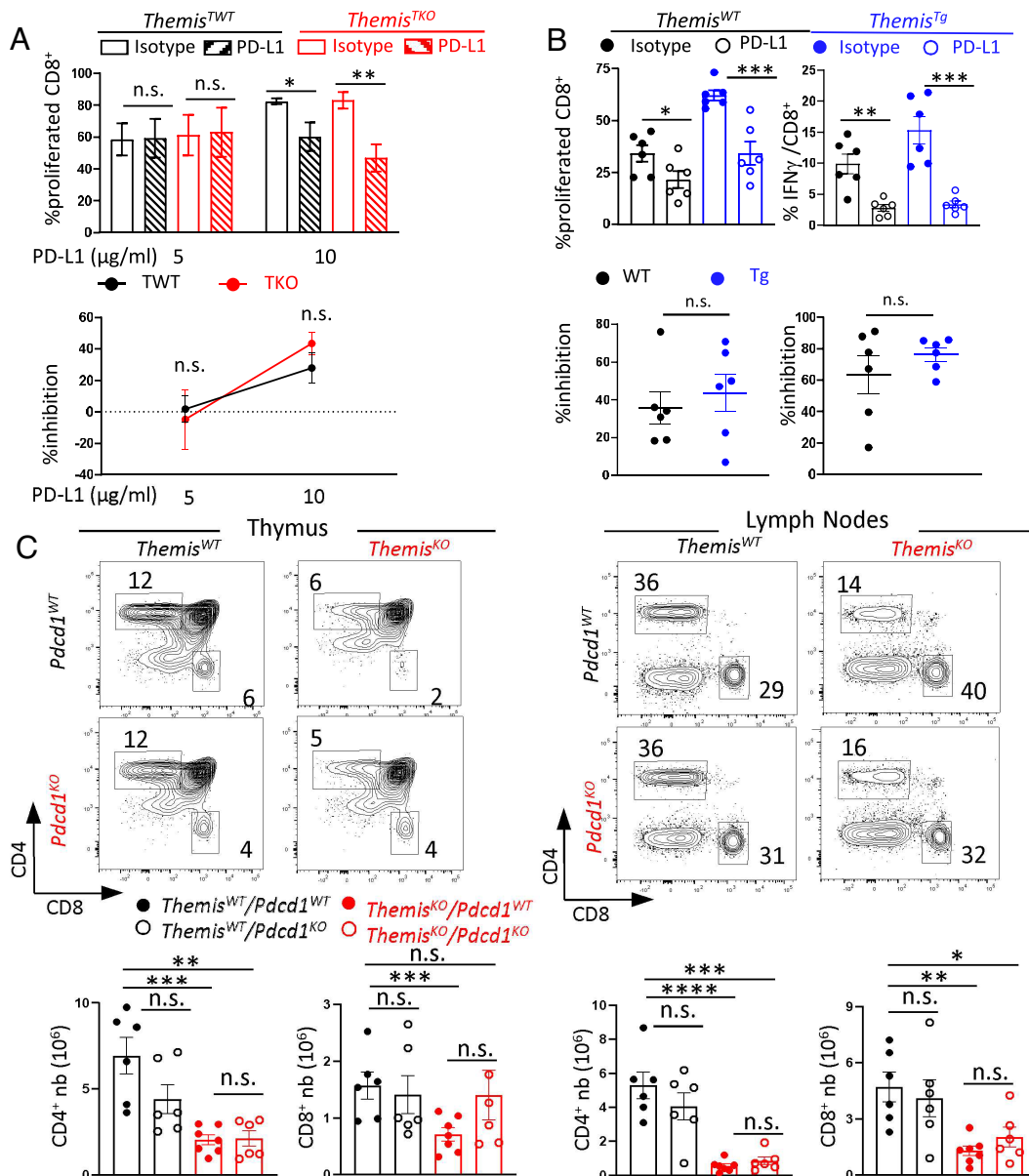


**Fig. 3.** Deletion of BTLA restores thymocyte positive selection in *Themis*<sup>KO</sup> mice. (A) Dot plots show CD4 versus CD8 surface staining on thymocytes from *Themis*<sup>WT</sup>*Btla*<sup>WT</sup>, *Themis*<sup>KO</sup>*Btla*<sup>WT</sup>, *Themis*<sup>WT</sup>*Btla*<sup>KO</sup>, and *Themis*<sup>KO</sup>*Btla*<sup>KO</sup> mice. (B) Dot plots show CD4 versus CD8 surface staining on Va2<sup>+</sup> thymocytes and total peripheral T cells from *Themis*<sup>WT</sup>*Btla*<sup>WT</sup>, *Themis*<sup>KO</sup>*Btla*<sup>WT</sup>, *Themis*<sup>WT</sup>*Btla*<sup>KO</sup>, and *Themis*<sup>KO</sup>*Btla*<sup>KO</sup> mice expressing the transgenic TCR OT-1. Bars graphs represent the numbers and percentages of Va2<sup>+</sup> CD8<sup>+</sup> thymocytes and lymph nodes T cells from mice of the indicated genotype. Data are mean ± SEM and represent a pool of five independent experiments each including n = 1 to 2 mice per group. Data were analyzed by the unpaired two-tailed Mann-Whitney *t* test. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, and \*\*\*\**P* < 0.0001.

mature TCR+CD62L<sup>low</sup> CD8<sup>+</sup> SP thymocytes, were increased in *Themis*<sup>KO</sup>*Pdcd1*<sup>KO</sup> mice to nearly reach the values observed in *Themis*<sup>WT</sup>*Pdcd1*<sup>WT</sup> mice (Fig. 4C and SI Appendix, Fig. S4A). The deletion of *Pdcd1* mildly increased the surface expression of CD5 on *Themis*<sup>KO</sup> thymocytes which remained far below the levels observed in *Themis*<sup>WT</sup>*Pdcd1*<sup>WT</sup> mice (SI Appendix, Fig. S4C). Together, these data indicated that the deletion of *Pdcd1* did not correct the defect of CD4<sup>+</sup> T cell development and TCR signaling in THEMIS-deficient thymocytes but could elicit CD8<sup>+</sup> T cell development, possibly by mildly increasing the intensity of TCR signals. In peripheral lymphoid organs, the percentages and numbers of CD8<sup>+</sup> T cells in *Themis*<sup>KO</sup>*Pdcd1*<sup>KO</sup> mice remained similar to that in *Themis*<sup>KO</sup>*Pdcd1*<sup>WT</sup> mice (Fig. 4C), showing that the loss of PD-1 incompletely rescued the defects affecting CD8<sup>+</sup> T cells in *Themis*<sup>KO</sup> mice. Collectively, these results suggested that the defect of T cell development in *Themis*<sup>KO</sup> mice does not result from exacerbated PD-1-dependent inhibitory signals.

**Restriction of BTLA Inhibitory Function by THEMIS Promotes the Homeostatic Maintenance of Naive CD8<sup>+</sup> T cells.** BTLA is expressed at low levels on naive CD8<sup>+</sup> T cells and regulates their homeostasis, as revealed by the ability of BTLA to restrain proliferation of naive CD8<sup>+</sup> T cells upon transfer into lymphocyte-deficient host (37). Mice containing a postthymic deletion of *Themis* in peripheral T cells have reduced numbers of naive CD8<sup>+</sup> T cells in peripheral lymphoid organs (18, 21). *Themis* postthymic deletion also decreases the potential of naive CD8<sup>+</sup> T cells to proliferate into lymphocyte-deficient hosts (18), suggesting that the restriction of BTLA signaling by THEMIS could enhance the

maintenance of CD8<sup>+</sup> T cells. To address this possibility, we crossed postthymic THEMIS-deficient mice (*Themis*<sup>TKO</sup>) with *Btla*<sup>KO</sup> mice to evaluate whether the functional inactivation of BTLA could rescue the defect of CD8<sup>+</sup> T cell homeostasis associated with the loss of THEMIS. As already established (18), numbers of peripheral CD8<sup>+</sup> T cells were reduced in *Themis*<sup>TKO</sup>*Btla*<sup>WT</sup> mice in comparison to that in control *Themis*<sup>TWT</sup>*Btla*<sup>WT</sup> mice, whereas numbers of CD4<sup>+</sup> T cells remained unchanged (Fig. 5A). The deletion of *Btla* in THEMIS-sufficient mice did not significantly modify the numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Fig. 5A). However, numbers of CD8<sup>+</sup> T cells in *Themis*<sup>TKO</sup>*Btla*<sup>KO</sup> mice were restored to values comparable to those in *Themis*<sup>TWT</sup>*Btla*<sup>WT</sup> mice, indicating that the invalidation of BTLA rescued the defect of CD8<sup>+</sup> T cell maintenance in *Themis*<sup>TKO</sup> mice (Fig. 5A). To address whether THEMIS stimulates lymphopenia-induced proliferation of CD8<sup>+</sup> T cells by repressing BTLA, we transferred CellTrace Violet (CTV) stained CD45.1<sup>+</sup> CD8<sup>+</sup> T cells from *Themis*<sup>TKO</sup> or *Themis*<sup>TKO</sup>*Btla*<sup>KO</sup> mice mixed in a 1:1 ratio with CTV-stained CD45.2<sup>+</sup> CD8<sup>+</sup> T cells from C57BL/6 mice into *Rag*<sup>KO</sup>*γc*<sup>KO</sup> host mice which lack T, B, and NK cells (Fig. 5B). The percentage of *Themis*<sup>TKO</sup> CD8<sup>+</sup> T cells that proliferated were reduced in comparison to those in THEMIS expressing CD8<sup>+</sup> T cells (Fig. 5C and D). As opposed to CD8<sup>+</sup> T cells from wild-type mice, which were normally maintained, the percentages of CD45.1<sup>+</sup> cells from *Themis*<sup>TKO</sup> mice recovered in the spleen were decreased to 20% (Fig. 5E). The deletion of BTLA in the THEMIS-deficient background increased the proportions of CD8<sup>+</sup> T cells recovered in the spleen to 40% (Fig. 5E) and restored proliferative responses (Fig. 5C and D), which were comparable to those observed in



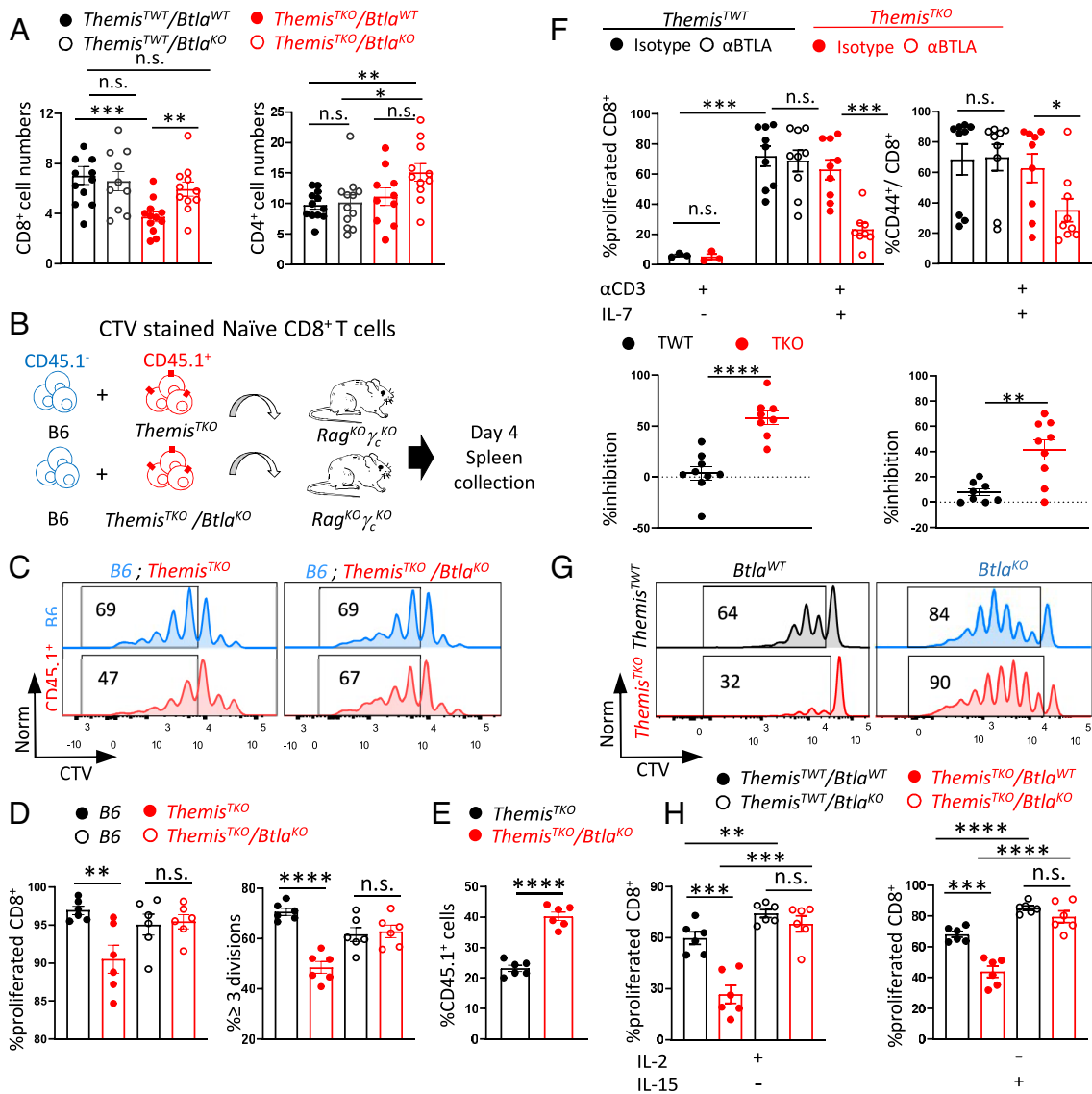
**Fig. 4.** THEMIS does not regulate the inhibitory functions of PD-1 in peripheral T cells and thymocytes. (A) Naive CD44-CD25- CD8<sup>+</sup> T cells from *Themis*<sup>TWT</sup> and *Themis*<sup>TKO</sup> mice were stained with CTV and stimulated with anti-CD3 and anti-CD28 antibodies in the presence of the indicated doses of plate-bound recombinant PD-L1-Fc proteins or isotype control antibody. Bar graphs represent the percentages of cells that proliferated as determined by flow cytometry at 72 h. Curve graphs represent the percentages of inhibition of proliferation by PD-1 in CD8<sup>+</sup> T cells from *Themis*<sup>TWT</sup> (black curves) and *Themis*<sup>TKO</sup> (red curves) mice. Data are mean  $\pm$  SEM and represent two independent experiments each including n = 3 mice per group. (B) Naive CD44-CD25- CD8<sup>+</sup> T cells from *Themis*<sup>WT</sup> and *Themis*<sup>Tg</sup> mice were stained with CTV and stimulated with anti-CD3 and anti-CD28 antibodies in the presence of plate-bound recombinant PD-L1-Fc proteins or isotype control antibody. Bar graphs represent the percentages of cells that proliferated and produced IFN $\gamma$  as determined by flow cytometry at 72 h. Dot graphs represent the percentages of inhibition of each response by PD-1 in CD4<sup>+</sup> T cells from *Themis*<sup>WT</sup> (black dots) and *Themis*<sup>Tg</sup> (blue dots) mice. Data are mean  $\pm$  SEM and represent two independent experiments each including n = 3 mice per group. (C) Dot plots show CD4 versus CD8 surface staining on thymocytes from *Themis*<sup>WT</sup>Pdccl1<sup>WT</sup>, *Themis*<sup>TKO</sup>Pdccl1<sup>WT</sup>, *Themis*<sup>WT</sup>Pdccl1<sup>KO</sup>, and *Themis*<sup>TKO</sup>Pdccl1<sup>KO</sup> mice. Bars graphs represent the numbers of CD4<sup>+</sup> and CD8<sup>+</sup> thymocytes and lymph nodes T cells from mice of the indicated genotype. Data are mean  $\pm$  SEM and represent a pool of three independent experiments each including n = 2 to 3 mice per group. Data were analyzed by the unpaired two-tailed Mann-Whitney *t* test. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, and \*\*\*\**P* < 0.0001.

wild-type cells. Together, these results demonstrated that the attenuation of BTLA signaling by THEMIS is required for the optimal maintenance of a naive CD8<sup>+</sup> T cell pool.

Homeostatic expansion of CD8<sup>+</sup> T cells depends on the interaction of the TCR with low-affinity ligands and on signals driven by cytokines such as IL-7 (38). To better characterize the mechanism by which BTLA regulates the homeostatic expansion of CD8<sup>+</sup> T cells and evaluate the effect of THEMIS on this process, we stimulated naive CD8<sup>+</sup> T cells from *Themis*<sup>TWT</sup> and *Themis*<sup>TKO</sup> mice with suboptimal doses of plate-bound anti-CD3 antibodies together with IL-7. In this model, stimulation of the TCR induced slow proliferative responses which could be detected after 14 d of

culture and only when the TCR was engaged in the presence of IL-7 (Fig. 5F). The stimulation of BTLA on *Themis*<sup>TWT</sup> CD8<sup>+</sup> T cells had no detectable effects on the proliferation and on the expression of the activation marker CD44 which is induced following homeostatic expansion (39) (Fig. 5F). In contrast, proliferation and CD44 expression were strongly attenuated when BTLA was stimulated on *Themis*<sup>TKO</sup> CD8<sup>+</sup> T cells (Fig. 5F), showing that BTLA could inhibit proliferation induced by weak TCR signals when THEMIS-deficient CD8<sup>+</sup> T cells were cultured with IL-7.

It has been suggested that THEMIS could enhance the homeostatic expansion of CD8<sup>+</sup> T cells by positively regulating signals



**Fig. 5.** Deletion of BTLA restores TCR- and cytokine-driven peripheral CD8<sup>+</sup> T cell maintenance in *Themis*<sup>TKO</sup> mice. (A) Bar graphs represent the numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells from *Themis*<sup>TWT</sup>/*Btla*<sup>WT</sup>, *Themis*<sup>TWT</sup>/*Btla*<sup>KO</sup>, *Themis*<sup>TKO</sup>/*Btla*<sup>WT</sup>, and *Themis*<sup>TKO</sup>/*Btla*<sup>KO</sup> mice. Data are mean  $\pm$  SEM and represent six independent experiments each including  $n = 1$  to 2 mice per group. (B) Scheme representing the experimental setting for data shown in (C–E). Naïve CD45.2<sup>+</sup>CD8<sup>+</sup> T cells from C57BL/6 mice and naïve CD45.1<sup>+</sup>CD8<sup>+</sup> T cells from *Themis*<sup>TKO</sup> or *Themis*<sup>TKO</sup>/*Btla*<sup>KO</sup> mice were stained with CTV and mixed in a 1:1 ratio prior transfer into *Rag*<sup>KO</sup> $\gamma_c$ <sup>KO</sup> mice. Splens were collected at day 4 after transfer, and the isolated cells were analyzed by flow cytometry. (C) The histogram graphs show the CTV dilution in C57BL/6, *Themis*<sup>TKO</sup>, and *Themis*<sup>TKO</sup>/*Btla*<sup>KO</sup> CD8<sup>+</sup> T cells isolated from *Rag*<sup>KO</sup> $\gamma_c$ <sup>KO</sup> mice. (D) Bar graphs represent the percentages of cells that divided at least one time or that divided more than three times as represented by the gate in the CTV dilution histogram graph in (C). Data are mean  $\pm$  SEM and represent two independent experiments each including  $n = 3$  mice per group. (E) Bar graphs represent the percentages of *Themis*<sup>TKO</sup> and *Themis*<sup>TKO</sup>/*Btla*<sup>KO</sup> CD45.1<sup>+</sup> T cells among CD8<sup>+</sup> T cells recovered in the spleen of *Rag*<sup>KO</sup> $\gamma_c$ <sup>KO</sup> mice at day 4 after cotransfer. Data are mean  $\pm$  SEM and represent two independent experiments each including  $n = 3$  mice per group. (F) Naïve CD44<sup>+</sup>CD25<sup>+</sup>CD8<sup>+</sup> T cells from *Themis*<sup>TWT</sup> and *Themis*<sup>TKO</sup> mice were stained with CTV and stimulated for 14 d with anti-CD3 (1  $\mu$ g/mL) and anti-CD28 antibodies in the presence of plate-bound anti-BTLA or isotype control antibodies in cell-culture medium containing or not IL-7. Bar graphs represent the percentages of cells that divided at least one time and the surface expression of CD44 as determined by flow cytometry after 14 d of culture. Dot graphs represent the percentages of inhibition of proliferation and CD44 surface expression by BTLA in CD8<sup>+</sup> T cells from *Themis*<sup>TWT</sup> (black dots) and *Themis*<sup>TKO</sup> (red dots) mice. Data are mean  $\pm$  SEM and represent three independent experiments each including  $n = 3$  mice per group. (G) Naïve CD44<sup>+</sup>CD25<sup>+</sup>CD8<sup>+</sup> T cells from *Themis*<sup>TWT</sup>/*Btla*<sup>WT</sup>, *Themis*<sup>TKO</sup>/*Btla*<sup>WT</sup>, *Themis*<sup>TWT</sup>/*Btla*<sup>KO</sup>, and *Themis*<sup>TKO</sup>/*Btla*<sup>KO</sup> mice were stained with CTV and cultured for 7 d in cell-culture medium containing IL-15. The histogram graphs show the CTV dilution in CD8<sup>+</sup> T cells from the indicated genotypes. (H) Bar graphs represent the percentages of CD8<sup>+</sup> T cells from the indicated genotypes that divided at least one time after 7 d of culture with IL-2 or IL-15. Data are mean  $\pm$  SEM and represent two independent experiments each including  $n = 3$  mice per group. Data were analyzed by the unpaired two-tailed Mann-Whitney *t* test. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, and \*\*\*\**P* < 0.0001.

driven by IL-2 and IL-15 in the absence of additional TCR costimulation (40). The ability of naïve CD8<sup>+</sup> T cells to proliferate in vitro in response to these cytokines is decreased in the absence of THEMIS and is restored in THEMIS-deficient cells upon deletion of the gene encoding SHP-1 (40). We thus hypothesized that BTLA might also have the capacity to inhibit proliferation induced by IL-2 and IL-15 and that the proliferative defects of THEMIS-deficient CD8<sup>+</sup> T cells might be the consequence of an increased signaling activity of BTLA. To address this possibility, we stimulated naïve CD62L<sup>hi</sup>CD44<sup>low</sup> CD8<sup>+</sup> T cells

from *Themis*<sup>TWT</sup>/*Btla*<sup>WT</sup>, *Themis*<sup>TKO</sup>/*Btla*<sup>WT</sup>, *Themis*<sup>TWT</sup>/*Btla*<sup>KO</sup>, and *Themis*<sup>TKO</sup>/*Btla*<sup>KO</sup> mice with IL-2 and IL-15 and analyzed their proliferative responses after 7 d of stimulation. Confirming previous findings, we observed that *Themis*<sup>TKO</sup>/*Btla*<sup>WT</sup> CD8<sup>+</sup> T cells proliferated less than *Themis*<sup>TWT</sup>/*Btla*<sup>WT</sup> CD8<sup>+</sup> T cells in response to IL-2 and IL-15 (Fig. 5 G and H). Noticeably, the deletion of BTLA in THEMIS-sufficient CD8<sup>+</sup> T cells increased their capacity to proliferate in response to those cytokines, indicating that BTLA inhibited proliferation induced by IL-2- and IL-15 without exogenous agonistic stimulation (Fig. 5 G and H). HVEM was

expressed on *Themis*<sup>TWT</sup>*Btla*<sup>WT</sup> and *Themis*<sup>TWT</sup>*Btla*<sup>KO</sup> CD8+ T cells during expansion (SI Appendix, Fig. S6A), suggesting that BTLA inhibits cytokine-mediated proliferation by engaging HVEM through cis- or trans-interaction on CD8+ T cells. The increased expansion of *Themis*<sup>TWT</sup>*Btla*<sup>KO</sup> CD8+ T cells was not caused by an inappropriate enrichment of memory CD44<sup>hi</sup>CD62L<sup>low</sup> CD8+ T cells (SI Appendix, Fig. S6 B and C), which are more responsive to IL-15 (41). Confirming previous studies, the deletion of BTLA had no detectable effect on proliferation, when naive CD8+ T cells were stimulated with plate-bound anti-CD3 antibodies (SI Appendix, Fig. S6D). Most importantly, the absence of THEMIS in BTLA-deficient background did not decrease the ability of cells to proliferate in contrast to what we observed in cells expressing BTLA, indicating that THEMIS enhances IL-2- and IL-15-driven proliferation by restraining the signaling capacity of BTLA (Fig. 5 G and H). Collectively, these data indicated that THEMIS facilitated the homeostatic expansion of naive CD8+ T cells by repressing the capacity of BTLA to inhibit signals driven by the TCR and by cytokines such as IL-2 and IL-15.

## Discussion

Immune checkpoints are required to raise the threshold of T cell activation and differentiation and reduce T cell responsiveness to self-antigens which could cause autoimmunity. However, it is unclear how TCR signals elicited by self-ligands escape from immune checkpoint-mediated inhibitory signals in the context of thymocyte positive selection and peripheral T cell maintenance. Here we have shown that a critical function of THEMIS is to enhance TCR signals required for positive selection and peripheral CD8+ T cell homeostasis by repressing the inhibitory signaling capacity of BTLA. THEMIS is recruited to BTLA together with GRB2, which interacts directly with the cytoplasmic tail of BTLA (27), and the tyrosine phosphatase SHP-1. The association of THEMIS with SHP-1 enhances or stabilizes the oxidation of the catalytic cysteine of SHP-1, leading to inhibition of its phosphatase activity (42).

Whether THEMIS operates as a positive or negative regulator of TCR signaling has been extensively debated (15, 23, 43). The phenotype of THEMIS-deficient mice (14, 16, 17, 22) and studies based on TCR signaling reporter mice (21) support mostly a stimulatory effect of THEMIS on TCR signaling in vivo. However, TCR stimulation on DP thymocytes by cognate pMHC tetramers (44) and on peripheral CD4+ T cells polarized in T<sub>H</sub>1 conditions by anti-CD3 antibodies (22) suggest that THEMIS inhibits TCR signaling when the TCR is stimulated in vitro independently of additional costimuli. One possible explanation for this apparent discrepancy could rely on the different contributions of SHP-1 and SHP-2 to the regulation of TCR signaling. Analysis based on SHP-1-deficient mice suggests that SHP-1 has a relatively mild impact on T cell responses in vitro upon TCR cross-linking (45–47). 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 (28). This suggests that SHP-1 might function more effectively in feedback loops triggered by inhibitory receptors, such as BTLA, which might enrich SHP-1 in proximity of TCR signaling complexes. 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 coinhibitory receptor stimulation. In contrast to SHP-1, which is mostly characterized as a negative regulator of TCR signaling, SHP-2 has been assigned more complex functions and was reported to have a positive

regulatory role on ERK1/2 activity upon pre-TCR or TCR cross-linking (25, 48). Therefore, the inhibitory effect of THEMIS on TCR signaling, which results more selectively in ERK1/2 inhibition (21, 22), could be the consequence of its effect on SHP-2. Thus, THEMIS might affect T cell responses differently according to whether BTLA is engaged or not with the TCR. Supporting this possibility, THEMIS was reported to repress CD8+ T cell differentiation into effector cells upon lymphocytic choriomeningitis viruses infection (49), which is associated with the down-regulation of HVEM surface expression on T cells and decreased BTLA-mediated inhibition of antiviral T cell responses (50).

Another controversy relative to the function of THEMIS pertains to its effect on SHP-1's PTP activity. Although it is generally accepted that THEMIS regulates thymocytes and T cells by modulating SHP-1's PTP activity, studies from different groups reported nearly opposite effects. Studies from Paul Love's lab suggested that THEMIS enhances TCR signaling and positive selection by stabilizing/promoting the oxidation of the catalytic cysteine of SHP-1, thereby repressing the PTP activity of SHP-1 (13). One strong evidence supporting this model was the fact that deleting SHP-1 at the DP stage, using a *Cd4-Cre* transgene, could restore normal numbers of SP thymocytes and peripheral T cells in *Themis*<sup>KO</sup> mice (13). Studies from other groups suggest that THEMIS inhibits TCR signaling by coactivating SHP-1 (51, 52). Here, the authors propose that THEMIS promotes T cell development by preventing the transmission of strong signaling events that might be inadequately triggered by self-ligands, possibly leading to misplaced negative selection. Here, the authors have failed to observe a developmental rescue of *Themis*<sup>KO</sup> thymocytes when SHP-1 was deleted using the same *Cd4-Cre* transgenic model (51). It was later suggested that small variation in the timing of SHP-1 deletion at the DP stage, when positive selection occurs, might result in different outcomes (33). To address this possibility, SHP-1 was deleted prior the DP stage using either *Lck-Cre* or *CD2-iCre* transgenes (33). Analysis of thymocyte subsets showed first that the deletion of SHP-1 and/or SHP-2 in mice failed to result in a phenotype resembling that of THEMIS-deficient mice (33), indicating that the defect of T cell development in the absence of THEMIS cannot be attributed to the reduced tyrosine phosphatase activities of SHP1, SHP2, or both. More importantly, in both models, the loss of SHP-1 at early stages of T cell development alleviates the developmental block of *Themis*<sup>-/-</sup> thymocytes (33), supporting that the defect observed in the absence of THEMIS is caused by enhanced rather than decreased SHP-1's PTP activity. The deletion of SHP-1 also rescues the maintenance of CD8+ T cells in mice with conditional deletion of THEMIS in peripheral T cells (18). In agreement with these experiments, we showed that the deletion of the BTLA encoding gene rescues the defect of positive selection in *Themis*<sup>KO</sup> mice and the defect of CD8+ T cell maintenance in *Themis*<sup>TKO</sup> mice. These results together with those showing that BTLA has enhanced inhibitory function in the absence of THEMIS strongly support that THEMIS operates by restraining the BTLA-SHP-1 signaling axis.

The deletion of BTLA attenuates the oxidation of SHP-1 on its catalytic cysteine, suggesting that THEMIS inactivates SHP-1 upon its recruitment to BTLA. The recruitment of THEMIS-GRB2 complexes to BTLA could be important to facilitate the interaction of THEMIS's CABIT modules with the phosphatase domain of SHP-1 or might be required for additional steps of regulation affecting the phosphorylation (53) or the ubiquitinylation (24) of THEMIS. This is in accordance with reports showing that GRB2 facilitates the inactivation of SHP-1 by THEMIS during T cell development (29). Other inhibitory receptors such as ILT-2 and LAIR-1 interact with SHP-1 in T cells (54, 55) but lack

conventional GRB2-binding motifs on their cytoplasmic tail, suggesting that SHP-1 associated with these receptors might possibly be less responsive to THEMIS-based regulation, although this requires additional investigation. PD-1 also recruits GRB2 together with SHP-2 to its cytoplasmic tail following stimulation by PD-L1 (11), yet we could not detect an effect of THEMIS on regulatory pathways dependent on PD-1, supporting previous studies that reported a moderate inhibitory effect of THEMIS on SHP-2 activity in comparison to that on SHP-1 (13). PD-1 predominantly recruits SHP-2, but it also has the capacity to recruit SHP-1 (11, 20), which can maintain a functional PD-1 inhibitory signaling in the absence of SHP-2 (11). Although we were unable to detect an effect of THEMIS on PD-1-mediated signaling, we cannot exclude the possibility that THEMIS could affect TCR signaling by repressing the function of PD-1 in a context-specific manner. This might provide an explanation for the partial rescue of CD8+ T cell development observed in *Pdcd1<sup>KO</sup>Themis<sup>KO</sup>* mice, which in contrast to CD4+ T cell development is less dependent on strong or sustained TCR signals (56).

The deletion of *Btla* in THEMIS-sufficient mice enhances the capacity of naive CD8+ T cells to expand upon transfer into lymphopenic hosts (37), suggesting that BTLA preserves part of its inhibitory functions in wild-type CD8+ T cells. The mechanism whereby BTLA regulates CD8+ T cell expansion in this context has remained ill-investigated. HVEM is expressed on immature dendritic cells (57) and on lymph nodes fibroblastic reticular cells (58), and may attenuate homeostatic signals driven by low-affinity TCR ligands and by IL-7 by engaging BTLA which is mildly expressed on naive T cells (32). Accordingly, BTLA strongly inhibited proliferation of THEMIS-deficient naive CD8+ T cells induced by weak TCR signals and IL-7. The deletion of BTLA in THEMIS-sufficient cells also enhanced the capacity of naive CD8+ T cells to proliferate in vitro in response to IL-2 and IL-15, which are important for the homeostatic maintenance of CD8+ T cells (40). Remarkably, we did not use agonist antibodies against BTLA or recombinant HVEM to stimulate BTLA in those assays, suggesting that BTLA might be directly stimulated in cis or in trans by HVEM expressed on CD8+ T cells (59). Deletion of BTLA could lead to an increase in IL-2/IL-15 receptor signaling which was shown to be negatively regulated by SHP-1 (40). Alternatively, the loss of BTLA could also enhance the costimulatory function of HVEM which is repressed by BTLA upon cis interaction with HVEM (60). Here, HVEM could be possibly stimulated by its other ligands such as CD160 or LIGHT which are expressed on the cell surface of CD8+ T cells (59).

What could be the biological advantage of repressing the signaling function of a receptor while maintaining its expression at the cell surface? BTLA is part of a complex ligands-receptors interaction network, which includes HVEM and additional receptors and ligands such as LIGHT, CD160, and lymphotoxin  $\alpha$  (61). BTLA interaction with HVEM triggers the propagation of nuclear factor-kappa B (NF- $\kappa$ B)-dependent signaling events downstream of HVEM that can have pleiotropic effects according to cell type (61, 62). The expression of signaling-inactive forms of BTLA at the membrane could possibly preserve its stimulatory function in response to HVEM and block the inhibitory outcomes resulting from BTLA engagement. The consequences of this in the context of T cell development is not clear since HVEM-deficient mice have no reported defect at this stage (63). However, it may contribute to explain why HVEM exerts anti-inflammatory effects in some disease models (63–65), presumably by stimulating inhibitory signals through BTLA, and proinflammatory effects in other models (66–69), possibly in contexts where BTLA signaling is incapacitated. The expression of BTLA and HVEM in the same cell leads

to the stable formation of a cis-complex in which BTLA suppresses HVEM-mediated signaling of NF- $\kappa$ B by all of its membrane-bound ligands (CD160, BTLA, and LIGHT) in trans (70). Thus, preserving BTLA expression in this context might also prevent the transmission of inappropriate HVEM-mediated signals in T cells which could lead to exacerbated and damageable T cell responses.

Our findings suggest that the responsiveness of inhibitory receptors to their ligands could be governed by intracellular molecules whose expression levels vary across immune-cell subsets or activation states in order to promote or refrain specific immunological processes. Inhibitory effects similar to those described here for BTLA have been described for the inhibitory receptor TIM-3 whose signaling capacities are repressed by the adapter protein human leukocyte antigen B (HLA-B)-associated transcript (BAT-3) (71). The impact of this could be important in the context of immune checkpoint blockade immunotherapies since it implies that the efficacy of treatments in a given individual or cancer type could be dependent on the intrinsic responsiveness of immune checkpoints to stimulation which is set by such intracellular factors. This might explain why the efficacy of treatments can be uncoupled from the expression levels of the targeted receptors on immune cells in the tumor environment (5). It also provides a possible rationale to explain why T cells expressing multiple immune checkpoints could remain highly functional in the context of cancers (72, 73) and virus infections (74, 75). A better characterization of the intracellular mechanisms that regulate immune-checkpoint activity might allow a refinement of the predicting criteria of treatment efficacy and could possibly lead to the development of new approaches aiming at raising the signaling threshold of these receptors in complement to immune checkpoint blockade- or chimeric antigen receptor-based therapies.

## Materials and Methods

Complete experimental methods are described in *SI Appendix, Materials and Methods*.

**Mice.** *Themis<sup>KO</sup>* (16), *Themis<sup>Tg</sup>* (76), and *Cd2-Cre Themis<sup>fllox/fllox</sup>* (22) mice were described previously. *Btla<sup>KO</sup>* mice were kindly provided by T. Jacobs (77). *Pdcd1<sup>KO</sup>* and *Rag<sup>KO</sup> $\gamma_c$ <sup>KO</sup>* mice were from Jackson laboratories (strain #028276 and #014593, respectively). All of the experiments were conducted with sex- and age-matched mice between 6 and 14 wk old that were housed under specific pathogen-free conditions at the INSERM animal facility (Zootechnie US-006; accreditation number A-31 55508), which is accredited by the French Ministry of Agriculture to perform experiments on live mice. All experimental protocols were approved by the local ethics committee and are in compliance with the French and European regulations on the care and protection of laboratory animals (European Commission Directive 2010/63).

**Cell Purification and Cultures.** For T cell purification: Naive T cells from the spleen and lymph nodes were enriched by magnetic immunodepletion (Dynabeads from DB untouched mouse CD4 cells kit, Invitrogen) using the following monoclonal antibodies: anti-B220 (RA3-6-B2), anti-CD11b (M1/70), anti-MHC class II (m5-114), anti-Fc $\gamma$ Rs (2.4G2), NK1.1 (83PK136), anti-CD4 (GK1.5) or anti-CD8 (SN-H59) (generated in the lab) as well as anti-CD44 (IM7) and anti-CD25 (PC61) from BioXcell. For T cell stimulation: naive CD44-CD25- CD4+ and CD8+ T cells were stimulated in 96-well plates with variable amounts of plate-bound anti-CD3 antibodies (145-2C11, BioLegend) and 1  $\mu$ g/mL of soluble anti-CD28 antibodies (PV-1, BioLegend), in the presence of variable amounts of plate-bound anti-BTLA antibodies (PK18.6, BioXcell), HVEM-Fc recombinant proteins (BioLegend # 771306), PD-L1-Fc recombinant proteins (R&D systems, # 1019-B7) or rat IgG1 isotype control antibodies (BioXcell). For  $T_H$ 1 polarization assays: naive CD44-CD25- CD4+ T cells were stimulated as described above in the presence of IL-12 (10 ng/ml, PeproTech) and anti-IL-4 (10  $\mu$ g/ml, BVD4-1D11, BD Bioscience). For the analysis of cytokine production by flow cytometry, cells were restimulated for 4 h using Phorbol 12-myristate 13-acetate (PMA, 500 ng/ml), Ionomycin (1  $\mu$ g/ml) and Golgi-plug (1  $\mu$ g/ml) from BD Bioscience. For

proliferation assays: naive CD44-CD25- CD8+ T cells were labeled with 2  $\mu$ M CTV (Life Technologies) for 15 min at 37 °C and cultured in 96-well plates coated with anti-CD3 antibodies at the indicated doses. For experiments in Fig. 5, CTV-stained naive CD44-CD25- CD8+ T cells were cultured in 96-well plates coated with anti-CD3 antibodies at 1  $\mu$ g/mL in the presence of IL-7 (PeproTech, #217-17, 1 ng/mL) or with IL-2 (PeproTech, #212-12, 0.5 to 1  $\mu$ g/mL) or IL-15 (PeproTech, #210-15, 0.5 to 1  $\mu$ g/mL) alone. The incorporation of CTV was measured by flow cytometry after 7 and 14 d of culture.

**Analysis of TCR Signaling and Immunoprecipitation (IP).** All analyses were performed on CD4+ or CD8+ T cells that were shortly expanded to enable BTLA cell surface expression. Briefly, T cells were expanded for 48 h with plate-bound anti-CD3 (145-2C11, 5  $\mu$ g/mL) and soluble anti-CD28 (PV-1; 1  $\mu$ g/mL) antibodies from BioLegend. T cells were then harvested and grown in the presence of IL-2 (5 to 10 ng/mL, PeproTech) for 48 h. For IPs: A total of 0.2 to 1  $\times$  10<sup>8</sup> CD8+ T cells from C57Bl/6, *Themis*<sup>WT</sup>, *Themis*<sup>KO</sup>, and *Btla*<sup>KO</sup> mice were left untreated or treated with 100 nM pervanadate for 5 min at 37 °C. The treatment was stopped on ice, and cells were immediately centrifuged and resuspended in 1 mL of ice-cold lysis buffer [10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton, 2 mM Na3VO4, 5 mM NaF, 1 mM EDTA, 10 mM iodoacetamide, 10 mM N-Ethylmaleimide, and protease inhibitor cocktail tablet (Roche)] and incubated for 20 min on ice. Lysates were cleared by centrifugation at 18,000 g for 15 min at 4 °C, and SHP-1 or BTLA were subjected to IP from cleared lysates for 2 h at 4 °C with 15  $\mu$ L of protein A- and protein G-Sepharose resin coated with 10  $\mu$ g of polyclonal rabbit anti-SHP-1 antibodies (07-419, Millipore) or rat anti-BTLA antibodies (PK18.6, BioXcell), respectively. Resins were washed three times and incubated for 10 min at 95 °C with Laemmli buffer. For TCR signaling analysis: *Themis*<sup>WT</sup> and *Themis*<sup>KO</sup> CD8+ T cells were incubated with hamster anti-CD3-biot (145-2C11, BD Bioscience) and rat anti-BTLA (PK18.6, BioXcell) for 15 min on ice. Cells were washed and stimulated for the indicated time points with streptavidin and F(ab')<sub>2</sub> goat anti-rat IgG antibodies (Jackson ImmunoResearch). *Themis*<sup>WT</sup> and *Themis*<sup>tg</sup> CD4+ T cells were incubated with rat anti-mouse CD3 (17A2, BD Bioscience) and rat anti-BTLA (PK18.6, BioXcell) for 15 min on ice. Cells were washed and stimulated for the indicated time points with goat anti-rat IgG antibodies. Cell lysates were prepared as described above.

**Immunization of Mice with I-E $\alpha$  Peptide.** Mice were immunized subcutaneously on each side at the base of the tail with 40  $\mu$ g of peptide I-E $\alpha$  (EAWGALANKAVDKA from GeneCust) in CFA (Sigma-Aldrich). Anti-BTLA antibodies (clone PK18, BioXcell) or PBS were injected intraperitoneally (200  $\mu$ g/mouse) at the day of immunization and at days 3 and 6 postimmunization. CD4+ T cell populations from the draining lymph nodes were analyzed at day 9 after immunization. For staining of antigen-specific cells, I-E $\alpha$ -I-Ab tetramers were obtained from the NIH Tetramer core facility. Overall, cells at 1  $\times$  10<sup>8</sup>/mL were first stained at room temperature for 2 h with an optimal concentration of tetramer. Cell staining for additional markers was then performed as described earlier.

**Lymphopenia-Induced Proliferation.** Naive CD44-CD25- CD8+ T cells from C57Bl/6 (CD45.2+), *Themis*<sup>KO</sup> (CD45.1+), and *Themis*<sup>KO</sup>*Btla*<sup>KO</sup> (CD45.1+) mice were stained with CTV. 5  $\times$  10<sup>5</sup> CD8+ T cells from C57Bl/6 mice were then mixed in a 1:1 ratio with CD8+ T cells from *Themis*<sup>KO</sup> or *Themis*<sup>KO</sup>*Btla*<sup>KO</sup> mice and injected intravenously into *Rag*<sup>KO</sup>*yc*<sup>KO</sup> mice. Four days after transfer, splenic cells were collected, and CTV dilution was analyzed in CD45.1+ and CD45.2+ cells by flow cytometry.

**Data, Materials, and Software Availability.** All study data are included in the article and/or *SI Appendix*.

**ACKNOWLEDGMENTS.** We thank Loïc Dupré and Paul Love for helpful comments and suggestions and Dr. Thomas Jacobs for providing *Btla*<sup>-/-</sup> mice. We acknowledge the technical assistance provided by the personnel of INSERM US-006 Anexplo/Creffre animal facility. We thank Fatima-Ezzahra L'Faqih-Olive, Valérie Duplan-Eche, and Anne-Laure Iscache from the cytometry facility of INFINITY. This work was supported by INSERM; the Foundation ARSEP; the Association pour la Recherche sur le Cancer; the Agence Nationale de la Recherche (ANR-20-CE15-0002 and ANR-20-CE15-0005); SANOFI (iAward Europe); the French Ministry of Higher Education and Research (PhD fellowship for S.M.); and the China Scholarship Council (a PhD fellowship for C.Y.). Portions of this work were developed from the doctoral thesis of C.Y. (Université Paul Sabatier-Toulouse III, 2021) supervised by R.L.

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