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ARTICLE

Themis suppresses the effector function of CD8$^+$ T cells in acute viral infection

Jian Tang[1,2,7], Xian Jia[1,2,7], Jian Li[1,2,7], Junchen Dong[1,2,7], Jiayu Wang[1], Wanyun Li[1], Yuzhen Zhu[1,2], Yanyan Hu[1,2], Bowen Hou[1,2], Chunjie Lin[1,2], Yu Cong[1], Tong Ren[1], Changsheng Yan[1], Hongying Yang[1], Qian Lai[1], Haiping Zheng[1], Yuzhou Bao[1], Namrata Gautam[3,4], Hong-Rui Wang[2], Bing Xu[5], Xiao Lei Chen[1✉], Qing Li[1✉], Nicholas R. J. Gascoigne[3,4✉] and Guo Fu[1,5,6✉]

CD8$^+$ T cells play a central role in antiviral immune responses. Upon infection, naive CD8$^+$ T cells differentiate into effector cells to eliminate virus-infected cells, and some of these effector cells further differentiate into memory cells to provide long-term protection after infection is resolved. Although extensively investigated, the underlying mechanisms of CD8$^+$ T-cell differentiation remain incompletely understood. Themis is a T-cell-specific protein that plays critical roles in T-cell development. Recent studies using Themis T-cell conditional knockout mice also demonstrated that Themis is required to promote mature CD8$^+$ T-cell homeostasis, cytokine responsiveness, and antibacterial responses. In this study, we used LCMV Armstrong infection as a probe to explore the role of Themis in viral infection. We found that preexisting CD8$^+$ T-cell homeostasis defects and cytokine hyporesponsiveness do not impair viral clearance in Themis T-cell conditional knockout mice. Further analyses showed that in the primary immune response, Themis deficiency promoted the differentiation of CD8$^+$ effector cells and increased their TNF and IFNγ production. Moreover, Themis deficiency impaired memory precursor cell (MPEC) differentiation but promoted short-lived effector cell (SLEC) differentiation. Themis deficiency also enhanced effector cytokine production in memory CD8$^+$ T cells while impairing central memory CD8$^+$ T-cell formation. Mechanistically, we found that Themis mediates PD-1 expression and its signaling in effector CD8$^+$ T cells, which explains the elevated cytokine production in these cells when Themis is disrupted.

Keywords: Themis; Effector T cell; CD8 T-cell differentiation; Cytokine; LCMV

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INTRODUCTION

CD8$^+$ T cells play a central role in antiviral immune responses. Upon recognition of antigenic viral peptides presented by MHC-I molecules, naive CD8$^+$ T cells undergo a well-orchestrated process of activation and proliferation and differentiate into effector cells, which can effectively eliminate virus-infected cells [1]. After viral infection is resolved, most effector cells fade away; however, some effector cells further differentiate into memory cells, which can provide long-term protection upon reinfection by the same pathogen [2]. Historically, many molecules involved in T-cell effector function and memory formation were discovered or characterized by studying the mouse lymphocytic choriomeningitis virus (LCMV) infection model [3]. LCMV is a noncytolytic virus, and acute infection by the LCMV Armstrong (Arm) strain can be rapidly controlled within a week due to a robust T-cell-mediated immune response, particularly involving CD8$^+$ T cells. Therefore, LCMV Arm infection is a prototypical model to investigate the physiological function of a given molecule in CD8$^+$ T cells and the cellular and molecular mechanisms involved [4].

Themis is a protein specifically expressed in the T-cell lineage [5]. Using germline knockout mice [6–8] or ENU mutagenesis-derived mice [9–11], we and others previously reported that Themis deficiency or mutation can lead to a severe block in thymocyte development at the CD4$^+$8$^+$ double-positive stage. Moreover, a spontaneous mutation of *Themis* in rats impaired the function of regulatory T cells [12]. An interesting observation from the above studies is that chemically induced or spontaneously occurring mutations are not enriched in an apparent "hotspot" region but are instead distributed across the full sequence of Themis [9–12], suggesting that the structural integrity of Themis is essential for its function. While the importance of Themis in thymocyte development is well recognized, the molecular mechanisms involved in this process remain elusive. A common finding from multiple studies is that Themis constitutively binds tyrosine phosphatase SHP-1 [13–16], and hence, the functional

[1]State Key Laboratory of Cellular Stress Biology, School of Medicine, Faculty of Medicine and Life Sciences, Xiamen University, Xiamen, China. [2]School of Life Sciences, Xiamen University, Xiamen, China. [3]Immunology Translational Research Programme, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore. [4]Department of Microbiology and Immunology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore. [5]Department of Hematology, The First Affiliated Hospital and Institute of Hematology, School of Medicine, Xiamen University, Xiamen, China. [6]Cancer Research Center of Xiamen University, Xiamen, China. [7]These authors contributed equally: Jian Tang, Xian Jia, Jian Li, Junchen Dong. ✉email: cxl2015@xmu.edu.cn; sunnymaylq@xmu.edu.cn; micnrjg@nus.edu.sg; guofu@xmu.edu.cn

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importance of this interaction has been intensively investigated [13, 16, 17]. However, whether Themis positively or negatively regulates the activity of SHP-1 and hence TCR signaling in thymocytes is disputed [16, 17]. Themis is also associated with SHP-2 in thymocytes [13, 15–17], but the effect of Themis on SHP-2 is not known.

Compared to the extensive studies of Themis in thymocyte development, the function of Themis in mature peripheral T cells has just begun to be uncovered. A previous study reported that Themis is required for cerebral pathogenesis caused by malaria infection and can protect mice from pulmonary tuberculosis [11]. However, the mouse strain used in that study bears an ENU-induced germline mutation of Themis at the amino acid position 23 of Ile, hindering the interpretation of this result as a primary defect of peripheral T cells or a secondary effect due to impaired thymocyte development [11]. Additionally, mature T cells from germline Themis knockout mice show severe defects in their ability to respond to TCR stimulation by upregulating their metabolism. The lack of Themis affects the ability to induce insulin receptor upregulation [18]. Again, it is unclear whether or not this is an effect of impaired thymic differentiation. Recently, some of us and colleagues developed a *Themis* conditional knockout mouse strain in which *Themis* was specifically deleted in mature T cells by distal Lck-Cre (*Themis*$^{flox/flox}$-dLck-Cre, hereafter cKO) [19]. Using this strain, it was found that Themis is required to maintain CD8$^+$ T-cell homeostasis by integrating the signals derived from low-affinity TCR stimulation and cytokines [19]. Based on this finding, we further designed a "reductionist" experiment to circumvent TCR signaling, and showed that Themis is required for common gamma chain cytokine signaling, where the deficiency of Themis severely impaired CD8$^+$ T cells responding to IL-2 and IL-15 [20], two essential cytokines for CD8$^+$ T-cell expansion and differentiation [21]. The role of Themis in bacterial infection has also been explored in a *Listeria* infection model, where it was found that Themis is required for efficient CD8$^+$ T-cell responses [19]. Of note, it was shown that Themis-deficient CD8$^+$ T cells had a mild defect in IFNγ and TNF production when restimulated with high amounts of strong antigens but exhibited severe defects when restimulated with low amounts of the same antigens or with a high dose of a weak antigen. These results indicated that Themis is required to promote CD8$^+$ T-cell effector functions in response to low-affinity or low-avidity ligands but is relatively dispensable when responding to high-affinity ligands [19].

In this study, using LCMV Arm infection as a model, we found that despite a connate CD8$^+$ T-cell homeostasis defect and cytokine hyporesponsiveness, Themis cKO mice can clear LCMV Arm infection effectively. Moreover, in the primary immune response, we found that Themis-deficient CD8$^+$ T cells were more prone to cytokine production and favored the differentiation of short-lived effector cells (SLECs) over memory precursor effector cells (MPECs). In the memory stage, Themis-deficient CD8$^+$ T cells also exhibited a propensity to produce effector cytokines but suppressed central memory CD8$^+$ T-cell formation. In search for a mechanism of these findings, we found that Themis deficiency inhibited PD-1 expression and its downstream signaling in effector CD8$^+$ T cells, which could account for the elevated cytokine production in Themis-deficient CD8$^+$ T cells.

RESULTS

Homeostasis defects in Themis-deficient CD8$^+$ T cells do not impair viral clearance in LCMV Arm infection

Our previous study showed that Themis is required for CD8$^+$ T-cell homeostasis and that the number of CD8$^+$ T cells in the lymph nodes of Themis cKO mice was only half that of control mice (*Themis*$^{flox/flox}$, hereafter WT) [19]. We extended this finding and found similar results in the spleen (Fig. S1A, B) and lung (Fig. S1C, D) of Themis cKO mice, where the proportions and numbers of CD8$^+$ T cells were significantly reduced compared to those in WT mice. Moreover, this is a T-cell intrinsic defect because in a competitive cell cotransfer model, naive CD8$^+$ T cells derived from WT donor mice with an endogenous TCR repertoire consistently outnumbered their Themis cKO counterparts (Fig. S1E–G). On the basis of these findings, together with the previously reported impaired cytokine responsiveness of Themis-deficient CD8$^+$ T cells [20], we suspected that Themis cKO mice might have a severe defect in controlling a viral infection, such as LCMV, which mainly affects the spleen and lung [22, 23]. To investigate this, we infected Themis cKO and WT mice with the LCMV Arm strain. We found that all mice appeared normal, and histological examination revealed no signs of tissue damage or any noticeable differences in the spleens, lungs, kidneys, and livers between cKO and WT mice (Fig. 1A). When we examined the virus titer by a sensitive qPCR method [24], we found the virus level to be comparable in the serum of both strains of mice at 5 days post infection (dpi), whereas the virus was essentially cleared at 8 dpi (Fig. 1B). We then performed an in vivo killing assay to measure CD8$^+$ T-cell cytolytic activity, a function mainly dependent on perforin and granzyme B [25]. We found that the elimination of LCMV GP33-41 peptide-loaded target cells was equally efficient in WT and cKO mice (Fig. 1C). Together, these results demonstrated that Themis cKO mice can effectively control LCMV Arm infection despite their CD8$^+$ T cells bearing multiple severe defects from the start.

Themis restrains the differentiation of effector CD8$^+$ T cells

Next, we tried to understand how LCMV Arm infection was controlled in Themis cKO mice with preexisting CD8$^+$ T-cell defects. We first quantified the number of total CD8$^+$ T cells and found a lowered frequency and number of total CD8$^+$ T cells in the spleen of cKO mice at both 5 (Fig. S2A) and 8 dpi (Fig. 2A). Similar results were obtained in the lung (Fig. S2B, Fig. 2B). Interestingly, when we calculated the amplitude of expansion, we noted that the expansion rate was slightly increased in the spleen of Themis cKO mice compared to that in WT mice (Fig. 2C), and this phenomenon was even more pronounced in the lung compartment (Fig. 2D). These results were somewhat counterintuitive given the known defects in Themis cKO CD8$^+$ T cells but implied that Themis deficiency may confer compounded effects on CD8$^+$ T cells. To further investigate this, we examined the frequency of virus-specific effector CD8$^+$ T cells by staining MHC-I tetramers specific for LCMV epitopes (GP33-41, GP276-286, and NP396-404). At 5 dpi, only GP33-41$^+$ T cells were discernible in the spleen and lung of both strains of mice, whereas NP396-404$^+$ T cells were less abundant and GP276-286$^+$ T cells were barely detectable (Fig. S2C). In contrast, at 8 dpi, all three tetramer-positive cell populations were clearly identifiable (Fig. 2E).

We then compared the clonal expansion kinetics between WT and cKO mice. As previously reported [4, 26], with the virus being drastically controlled from 5 to 8 dpi (Fig. 1B), LCMV-specific CD8$^+$ T cells continuously expanded both proportionally (Fig. 2F) and numerically (Fig. 2G). Although the kinetics were roughly similar between WT and cKO mice, there were noticeable differences. For example, while the initial proportions of LCMV-specific T cells were comparable at 5 dpi, the frequencies of cKO-type cells increased to a higher level at 8 dpi, exceeding those of their WT counterparts (Fig. 2F). This elevated proportion of LCMV-specific CD8$^+$ T cells in cKO mice compensated for the severe reduction in their total CD8$^+$ T cells after infection (Fig. 2A, B) and consequently raised the number of LCMV-specific CD8$^+$ T cells in cKO mice to the level of their WT counterparts (Fig. 2G). These results suggested that Themis may have a dual role in effector CD8$^+$ T-cell differentiation. That is, on the one hand, Themis restrains the differentiation of effector CD8$^+$ T cells, such as in LCMV Arm infection, while on the other hand, it promotes their expansion and/or survival by mediating IL-2 and IL-15 signaling [20].

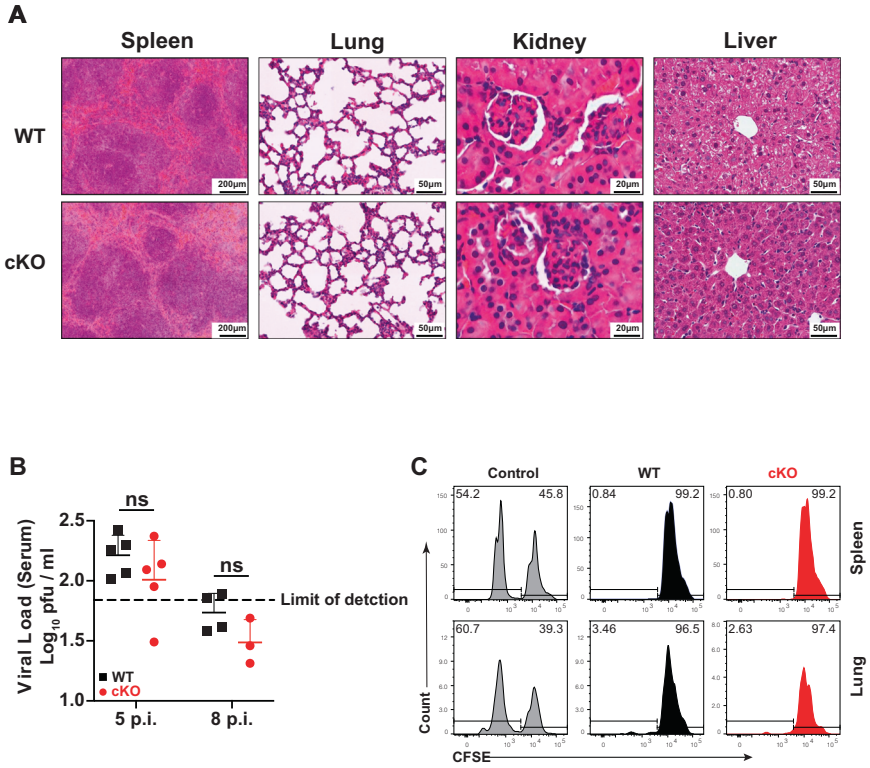

Fig. 1 LCMV Arm infection is controlled in Themis cKO mice. **A** H&E staining of tissue sections at 8 dpi. with the indicated magnification and region of interest. Scale bars as indicated, $n = 3$ independent biological repeats. **B** Virus titers determined by qPCR from the sera of infected mice. The dashed line indicates the limit of detection. Each symbol represents an individual mouse. For details, see Materials and Methods. Data shown (mean±sd) were from one of two experiments. **C** In vivo killing assay. LCMV GP33-41 peptide-loaded target splenocytes and no peptide-loaded control splenocytes were labeled with 0.25 μm and 5 μm CFSE, respectively, mixed in a 1:1 ratio, and cotransferred into LCMV Arm-infected mice at 8 dpi. Target cell elimination was determined by analyzing the CFSE profile 3 h post-transfer. $n = 2$ to 3 independent biological repeats. The *P* value was calculated by Student's *t* test and is shown in **B**

Themis suppresses effector cytokine production in CD8$^+$ T cells

Next, we wondered whether Themis may also affect the function of effector CD8$^+$ T cells when responding to LCMV Arm infection. In CD8$^+$ T cells, while granzyme B coupled with perforin mainly mediates cell-cell contact-dependent cytotoxicity [27], the non-cytolytic mechanisms mediated by IFNγ and TNF also contribute to viral clearance [28, 29]. Therefore, we examined these two branches of effector mechanisms on GP33-41$^+$ T cells as a representative of all LCMV-specific CD8$^+$ T cells. At 5 dpi, we found that the proportion and number of granzyme B-expressing cells were lower in the spleens of cKO mice than in those of WT mice (Fig. S3A, B). Moreover, the quantity of IFNγ-expressing and TNF/IFNγ coexpressing cells was also reduced in cKO mice (Fig. S3C, D). Similar patterns were observed in the lung (Fig. S3E–H). These results indicated that at this time point, cKO CD8$^+$ T cells were numerically and functionally inferior to their WT counterparts. In contrast, at 8 dpi, cKO CD8$^+$ T cells exhibited some superiority to their WT counterparts. For example, in the spleen, we found that the proportion of granzyme B-expressing cells was maintained at a higher level in cKO mice than in WT mice (Fig. 3A, B). Moreover, in cKO mice, there was a greater proportion of IFNγ-expressing and TNF plus IFNγ coexpressing cells (Fig. 3C, D). However, the increased proportion did not affect the gross number of granzyme B-producing or cytokine-expressing cells between WT and cKO mice (Fig. 3B, D, right). Similar proportional increases were also recorded in the lung (Fig. 3E–G), but this time, cKO mice presented more cytokine-expressing cells than their WT counterparts (Fig. 3H, right). Taken together, these results suggested that Themis may affect CD8$^+$ T-cell effector cytokine production in a time-dependent manner. That is, at the early stage (5 dpi), Themis promotes the production of cytokines, whereas at the late stage (8 dpi), Themis suppresses their production.

We were particularly interested in the inhibitory role of Themis in cytokine production at the late stage of infection, which was unexpected and contrasted with its stimulatory role in T-cell homeostasis and proliferation. Previously, we reported that Themis differentially regulated thymocyte responsiveness to peptide-MHC ligands with varied T-cell receptor (TCR) affinity [13]. We wondered whether this phenomenon existed in mature CD8$^+$ T cells and stimulated LCMV-specific effector CD8$^+$ T cells with a set of previously characterized GP33-41-derived altered peptides (A3V, L6F, W4Y) [30]. For splenic effector T cells, we found that the agonist peptide A3V, similar to its parental cognate peptide GP33-41, induced robust cytokine production (Fig. S4A). In proportion, there were more cKO CD8$^+$ T cells producing cytokines than their WT counterparts under these stimulations (Fig. S4B). Moreover, L6F seemed only to induce cytokine production in cKO CD8$^+$ T cells but not in WT cells, and W4Y barely induced any cytokine production (Fig. S4A, B). When we examined the effector T cells derived from the lung, we observed an essentially similar pattern (Fig. S4C, D). These results suggested that it is more likely a general effect for Themis to suppress effector cytokine production at the late stage of infection.

Themis deficiency impairs memory precursor effector cell differentiation but promotes short-lived effector cell differentiation

During LCMV Arm infection, whereas the majority of CD8$^+$ T cells acquired effector functions to fight off immediate infection and were short-lived upon elimination of infection, some effector

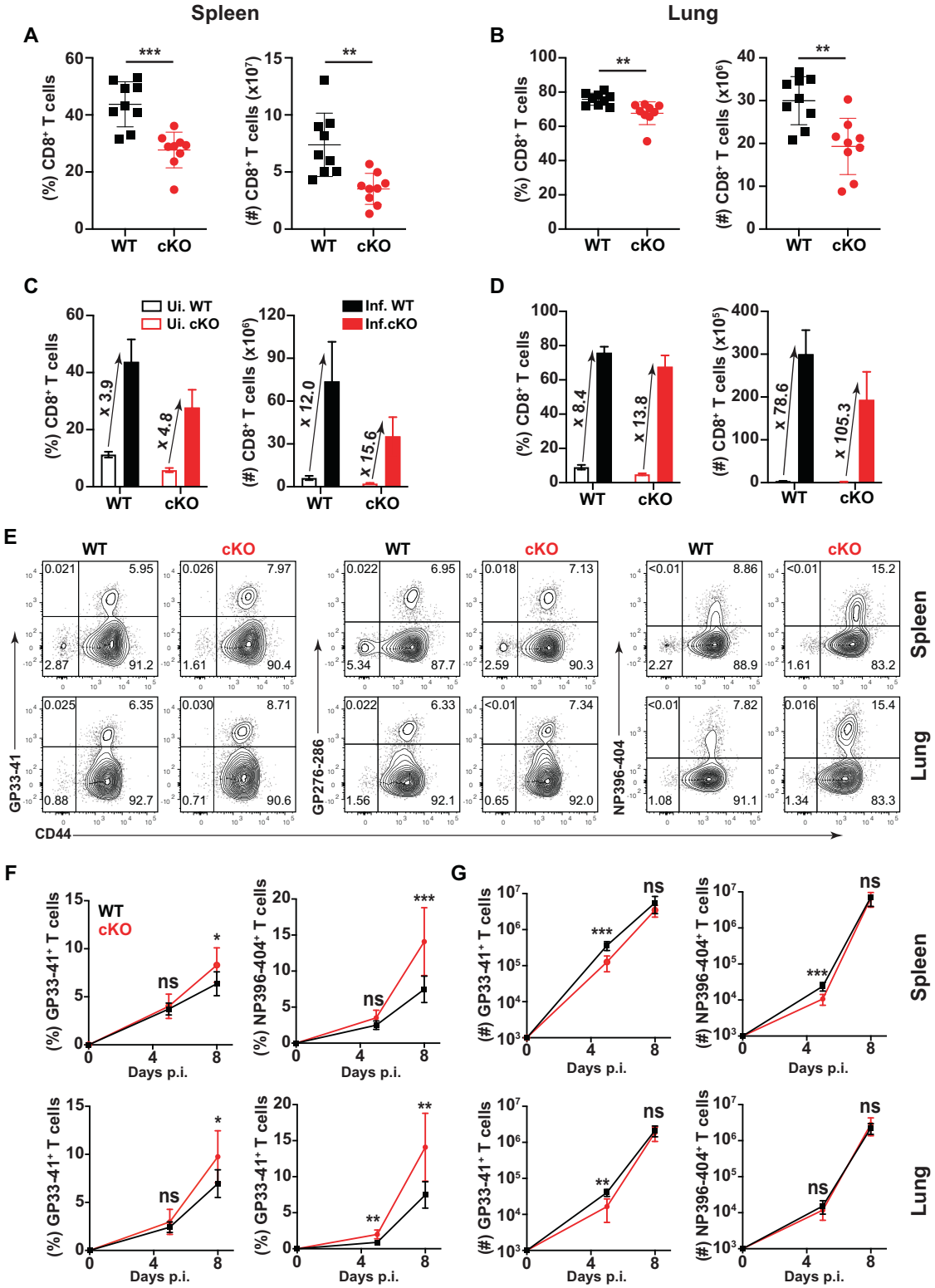

Fig. 2 Themis restrains the development of CD8$^+$ effector cells. WT and cKO mice were infected with LCMV Arm and analyzed at 8 dpi. **A**, **B** Summary of the frequency and number of total CD8$^+$ T cells in the spleen and lung. To calculate the expansion rate, the proportion and number of CD8$^+$ T cells at 8 dpi (values shown above in Panels **A** and **B**) were divided by those of CD8$^+$ T cells at steady-state (used values shown in Fig. S1B, D). The resultant expansion rates are indicated by solid lines with arrows for spleen (**C**) and lung (**D**). **E**–**G** Quantification of virus-specific CD8$^+$ T cells by the indicated tetramers. **E** Representative FACS plots are shown. Summary of the frequency (**F**) and number (**G**) of tetramer-positive CD8$^+$ T cells in the organs at the indicated times. The data shown are representative of one of three experiments at 8 dpi and one of two experiments at 5 dpi. Each symbol represents an individual mouse. *P* values were calculated by Student's *t* test (ns, nonsignificant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). The frequency and number of cells were artificially set to zero for the Day 0 time point in **F** and **G**, respectively

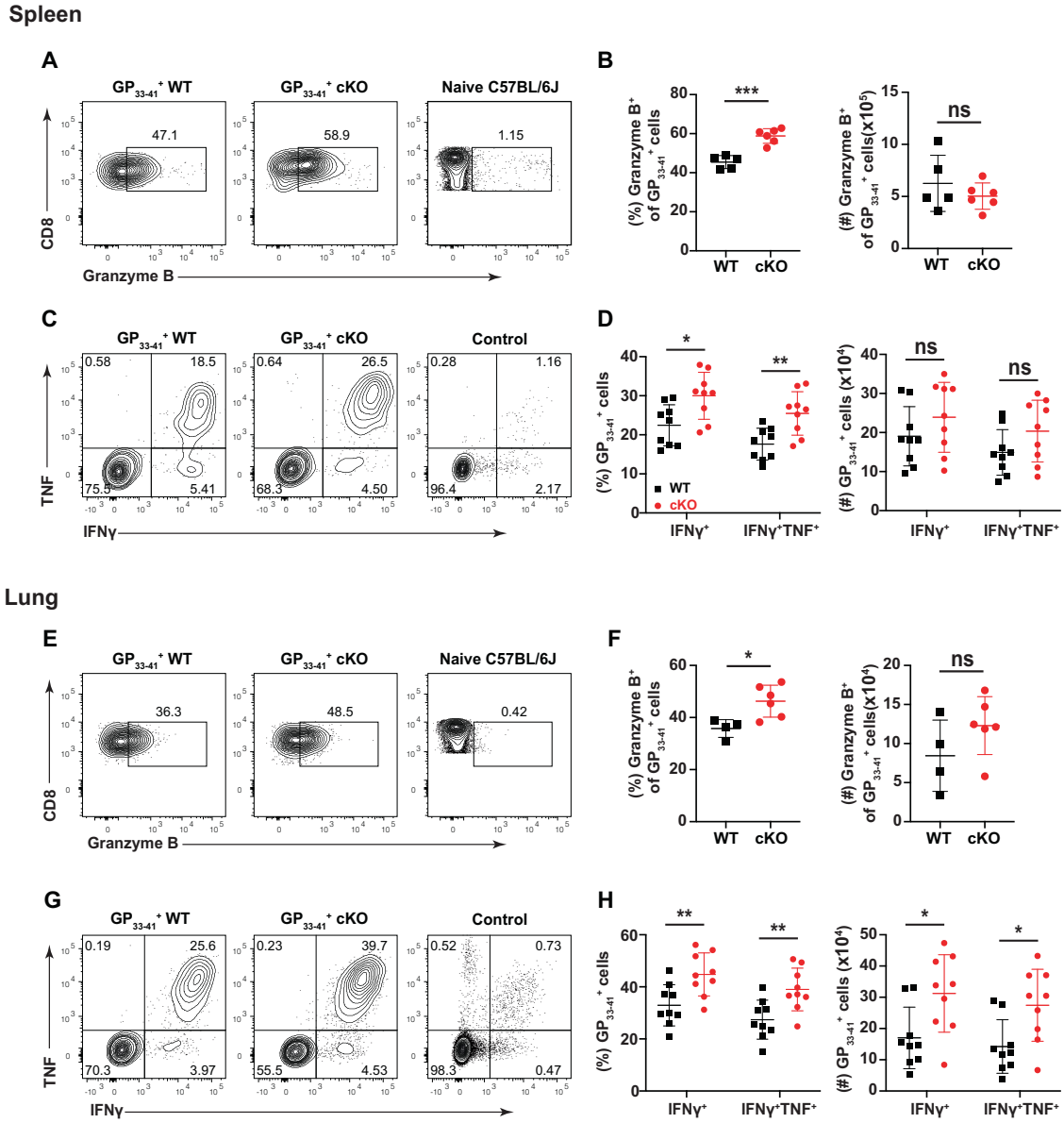

Fig. 3 Themis deficiency leads to increased effector function of CD8$^+$ T cells during the primary response. **A**–**D** Splenocytes obtained from WT and cKO mice were stimulated ex vivo with the LCMV GP33-41 peptide at 8 dpi. **A** Representative FACS plots of granzyme B staining. **B** Summary of the frequency (left) and number (right) of GP33-41$^+$ Granzyme B$^+$ cells. **C** Representative FACS plots of IFNγ and TNF production in GP33-41$^+$ cells. **D** Summary of the frequency (left) and numbers (right) of GP33-41$^+$ cytokine-expressing cells. **E**–**H** Lymphocytes from the lung were concurrently analyzed with the splenocytes in **A**–**D** and displayed in the same layout as for splenocytes. Data in **A**, **B**, **E**, **F** are from one experiment. Data in **C**, **D**, **G**, **H** are representative of two independent experiments. Each symbol represents an individual mouse. *P* values calculated by Student's *t* test (ns, nonsignificant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

CD8$^+$ T cells further differentiated into precursors of long-lived memory CD8$^+$ T cells, providing long-term immune protection [2]. These two types of effector CD8$^+$ T cells are termed short-lived effector cells (SLECs) and memory precursor effector cells (MPECs), respectively. They can be phenotypically distinguished by the reciprocal expression of KLRG1 and CD127, where KLRG1^{hi}CD127^{lo} demarcates SLECs and KLRG1^{lo}CD127^{hi} identifies MPECs [31]. We wondered whether Themis may play a role in the differentiation of SLECs versus MPECs and thus compared the expression of KLRG1 and CD127 on LCMV-specific CD8$^+$ T cells derived from Themis cKO or WT mice. We found that at 5 dpi, there was a clear population of SLECs (KLRG1$^{+/hi}$) in the spleen (Fig. S5A) and lung (Fig. S5B) of both strains of mice. Moreover, the proportion of SLECs was comparable between WT and cKO mice (Fig. S5C), but the absolute number of SLECs was lower in cKO mice (Fig. S5D). Of note, at this stage, MPECs (CD127$^{+/hi}$) were not discernible. In contrast, at 8 dpi, the MPEC population was more distinguishable in the spleen of both strains of mice (Fig. 4A). Moreover, we found that the proportion of SLECs was increased in cKO mice (Fig. 4B, top), whereas the proportion of MPECs was significantly reduced (Fig. 4B, bottom). However, when cell numbers were counted, there were fewer GP33-41$^+$ and comparable NP396-404$^+$ SLECs in cKO mice than in WT mice (Fig. 4C, top). As expected, the number of tetramer-positive MPECs was much lower in cKO mice than in WT mice (Fig. 4C, bottom). The expression of KLRG1 was also higher in cKO SLECs than in their WT counterparts (Fig. 4D). Similar results were obtained in the lung (Fig. 4E–H). Together, these results demonstrated that Themis is required to direct the differentiation away from predominantly SLECs and toward MPECs.

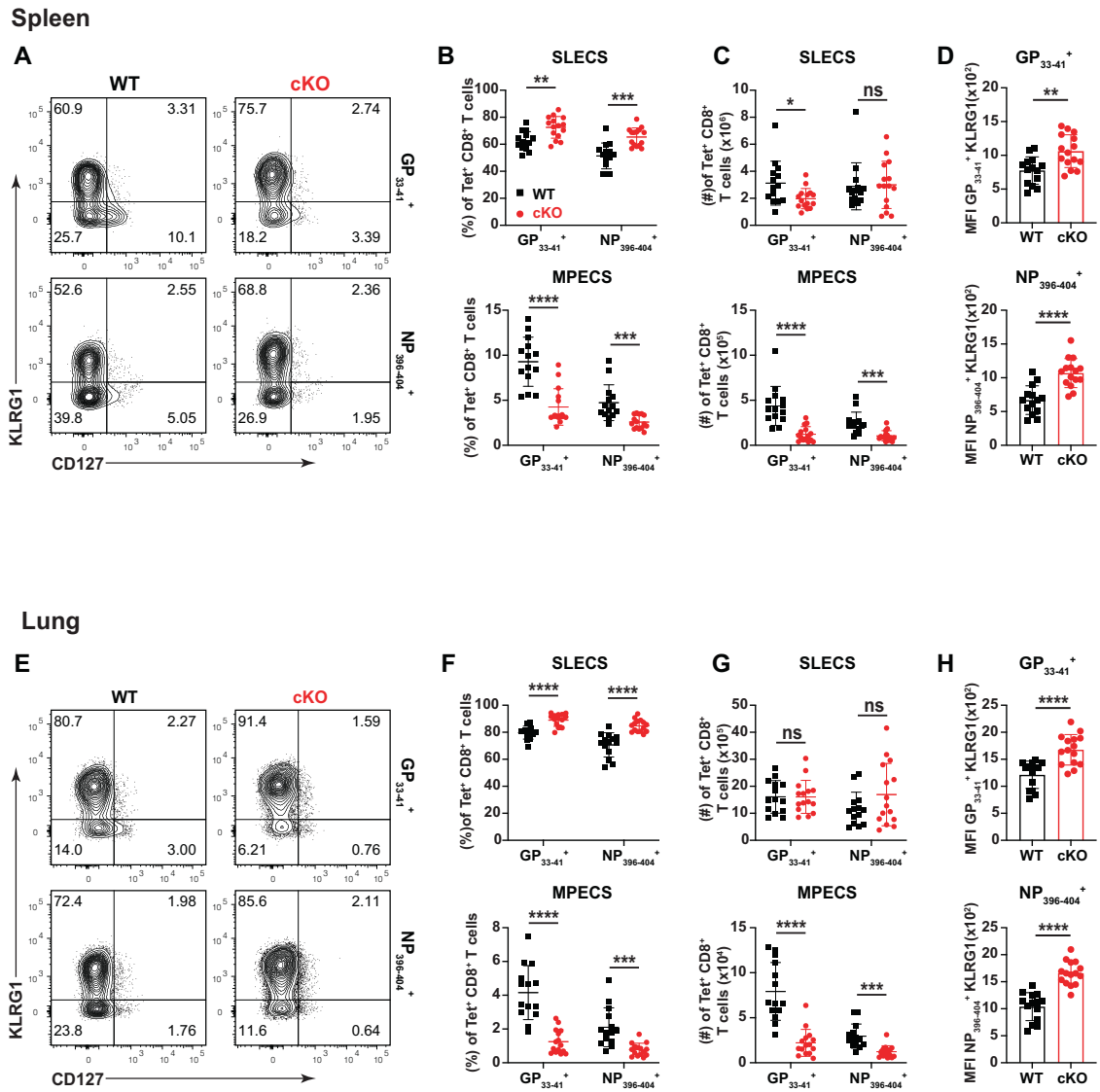

Fig. 4 Themis regulates SLEC and MPEC differentiation. **A**–**D** WT and cKO mice were infected with LCMV Arm for 8 days, and the subsets of SLECs ($CD127^{-}KLRG1^{+}$) and MPECs ($CD127^{+}KLRG1^{-}$) in the spleen were analyzed by FACS. **A** Representative FACS plots. **B** Summary of the frequency of SLECs (top) and MPECs (bottom). **C** Summary of the number of SLECs (top) and MPECs (bottom). **D** The mean fluorescence intensity (MFI) of KLRG1 in GP33-41^{+} (top) and NP396-404^{+} (bottom) CD8^{+} T cells. **E**–**H** Lymphocytes from the lung were concurrently analyzed with the splenocytes in **A**–**D** and displayed in the same layout as for splenocytes. **E** Representative FACS plots. **F**, **G** Summary of cell frequency and numbers of SLECs and MPECs as indicated. **H** MFI of KLRG1 in tetramer-positive CD8^{+} T cells as indicated. Data pooled from two experiments. Each symbol represents an individual mouse. *P* values were calculated by Student's *t* test (ns, nonsignificant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$)

Themis deficiency enhances effector cytokine production in memory CD8^{+} T cells

Our above results demonstrated that Themis deficiency could impair the differentiation of MPECs and further implied that there might be defects in the formation and function of memory CD8^{+} T cells. To test this hypothesis, we periodically sampled the blood of LCMV-infected mice and monitored the presence of LCMV-specific T cells. At 18 dpi, there was a clear population of GP33-41- and NP393-404-positive cells in both strains of mice (Fig. S6A). Proportionally, tetramer-positive cells were more abundant in cKO mice than in WT mice (Fig. S6B). This tendency was maintained at 30 dpi, although the frequency of tetramer-positive cells was decreased in both strains of mice (Fig. S6C, D). For more in-depth analysis, we aged the infected mice to more than 120 dpi, when the pool of memory CD8^{+} T cells was well established. Again, we found that the frequency of GP33-41- and NP396-404-positive cells was higher in cKO mice (Fig. 5A, B), although the absolute numbers of these tetramer-positive cells remained the same between cKO and WT mice (Fig. 5A, B). We next evaluated the functionality of these LCMV-specific memory CD8^{+} T cells by measuring their cytokine production. We detected an increased proportion and number of CD8^{+} T cells expressing IFNγ or coexpressing TNF and IFNγ in the spleen (Fig. 5C, D) and lung (Fig. 5E, F) of cKO mice. These results demonstrated that Themis deficiency also increased effector cytokine production in memory CD8^{+} T cells, as previously observed in effector CD8^{+} T cells.

Themis deficiency impairs central memory CD8^{+} T-cell formation

To ensure effective immunological memory, in addition to their number and functionality, the cellular composition of memory

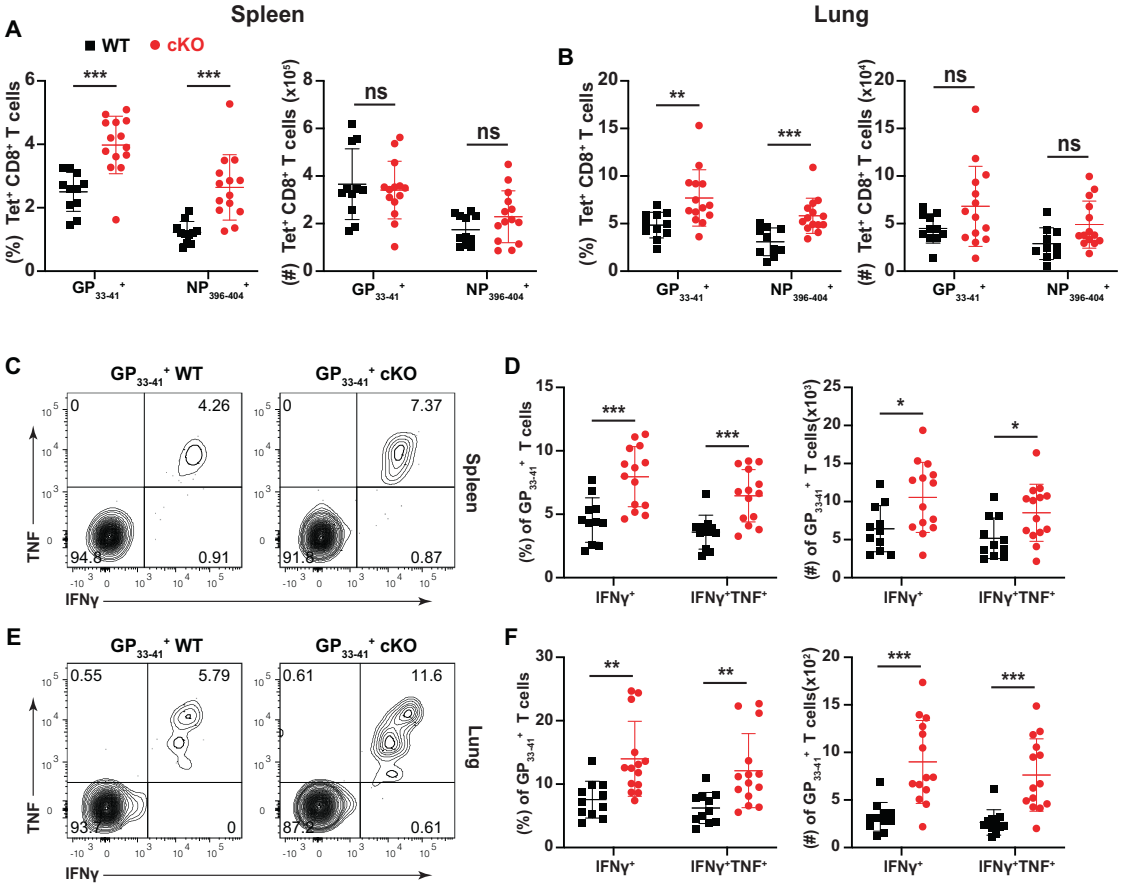

Fig. 5 Themis restrains the development of CD8$^+$ memory cells. WT and cKO mice were infected with LCMV Arm and analyzed at >120 dpi. **A** The frequency (left) and number (right) of virus-specific tetramer-positive CD8$^+$ T cells were analyzed in the spleen. **B** Lung samples were concurrently analyzed and displayed in the same layout as in **A**. **C** Representative FACS plots of cytokine production in GP33-41$^+$ cells in the spleen. **D** Summary of the frequency (left) and number (right) of GP33-41$^+$ cytokine-expressing cells as shown in **C**. **E**, **F** Lung samples were concurrently analyzed and displayed in the same layout as in **C**, **D**. Data were pooled from two experiments performed at 120 and 150 dpi. Each symbol represents an individual mouse. *P* values were calculated by Student's *t* test (ns, nonsignificant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

CD8$^+$ T cells also needs to be properly configured. It has been well established that memory CD8$^+$ T cells comprise two main subsets; whereas effector memory CD8$^+$ T cells (TEM) are preferentially localized in nonlymphoid tissue and exhibit immediate effector function, central memory CD8$^+$ T cells (TCM) are mainly localized in secondary lymphoid tissue and are more proliferative to provide sustained protection [4, 32, 33]. Phenotypically, TCM and TEM can be readily distinguished by the reciprocal expression of CD62L; TCM are CD62L^{hi} to facilitate their migration to secondary lymphoid organs, whereas TEM are CD62L^{lo} to promote their residence in inflamed tissues [34–36]. We found that at 70 dpi in the spleen, cKO mice had a comparable proportion of LCMV-specific TCM and TEM cells to their WT counterparts (Fig. S7A, B). In contrast, in the lung, cKO mice had a lower number of TCMs than WT mice (Fig. S7C, D). Previous studies have demonstrated that the representation of TCM increases with time after infection, while the percentage of TEM decreases [37–39]. We thus extended our observation to a later time point of 120 dpi, and consistent with previous reports, we found that the proportion of LCMV-specific TCM was increased in both strains of mice, whereas the frequency of TEM was reduced. Of note, at this time point, the frequency of TCM in Themis cKO mice was lower than that in WT mice in both the spleen (Fig. 6A, B) and lung (Fig. 6C, D). Taken together, these results demonstrated that Themis deficiency impaired TCM compartment formation within memory CD8$^+$ T cells.

We wanted to understand the reduction in TCM cells in Themis cKO mice because reportedly TCM cells have a greater capacity than TEM cells to persist in vivo due to their increased proliferative potential. Moreover, TCMs are more potent in mediating protective immunity, as exemplified in the LCMV clone 13 infection model [4]. LCMV clone 13 is a closely related viral strain to LCMV Arm and is known to cause systemic and chronic infection but shares GP33-41 and NP396-404 epitopes with the Arm strain [40]. Given the enhanced cytokine production on the one hand and the reduced TCM cells on the other hand, we wondered what the overall protection level would be in Themis cKO mice during a secondary infection. To answer this question, we rechallenged LCMV Arm-infected mice at 80 dpi with LCMV clone 13. At Day 5 post rechallenge, we found that the viral titers were comparable in the spleen and lung between WT and cKO mice (Fig. 6E). Quantitatively, while in cKO mice the proportion of virus-specific memory CD8$^+$ T cells was equal to (for GP33-41) or higher than (for NP396-404) that in WT mice (Fig. 6F, left), the sheer number of these cells was essentially the same between these two strains of mice (Fig. 6F, right). Functionally, we found that the production of IFNγ and TNF was slightly higher in cKO LCMV-specific GP33-41$^+$ T cells than in their WT counterparts (Fig. 6G). Together, these results demonstrated that although memory cKO CD8$^+$ T cells acquired some degree of advantage at the single-cell level (such as a higher proportion of NP396-404$^+$ T cells and elevated IFNγ and TNF production in GP33-41$^+$ T cells), these advantages were cancelled by the numerical disadvantage of long-lived TCM cells in Themis cKO mice, which resulted in a similar memory

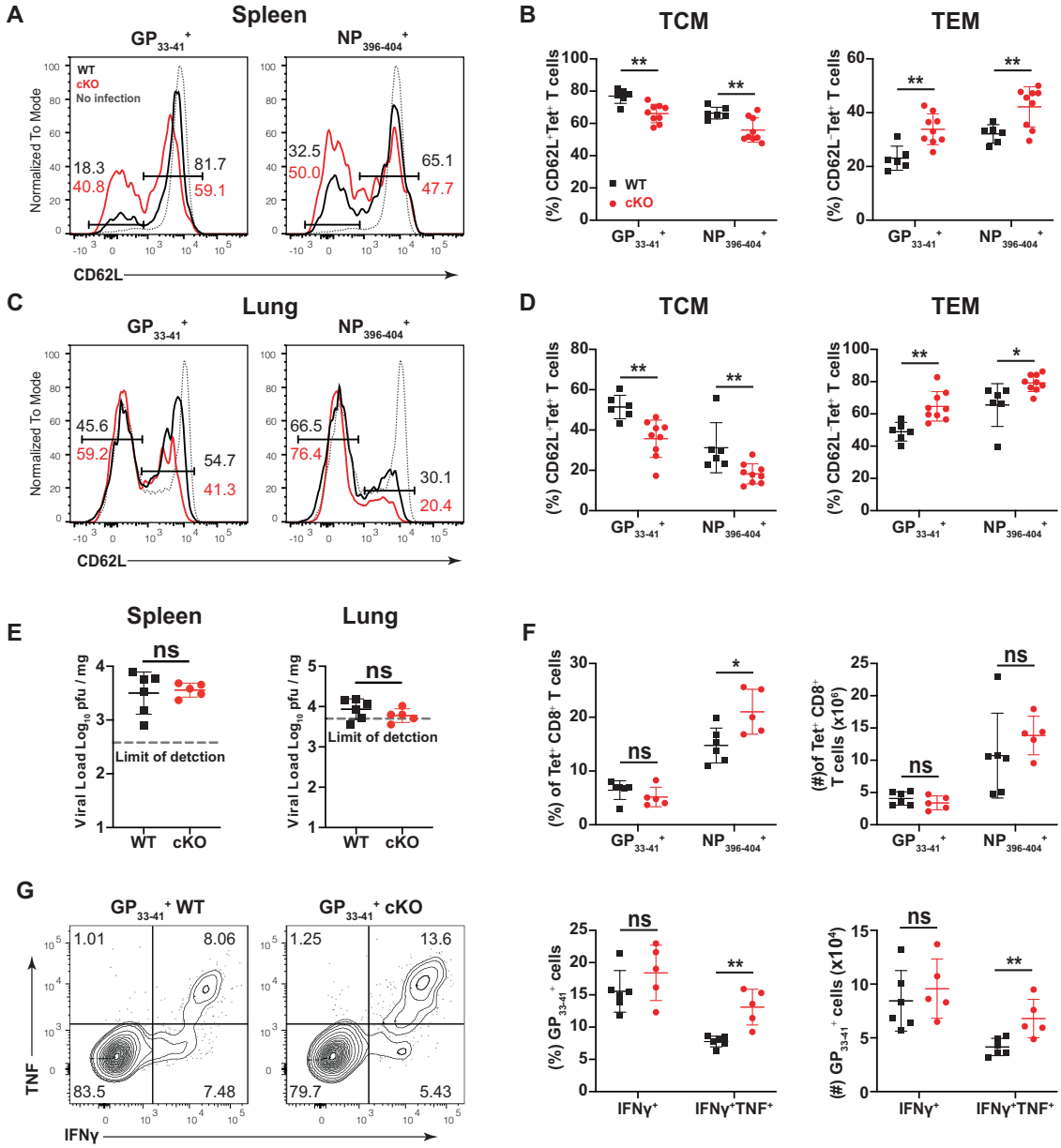

Fig. 6 Themis controls TCM formation. WT and cKO mice were infected with LCMV Arm and analyzed at 120 dpi. **A** Representative FACS plots of CD62L expression on virus-specific tetramer-positive $CD8^+$ T cells in the spleen. Values indicate the proportion of gated cells. **B** Summary of the frequency of TCM ($CD62L^+$, left) and TEM ($CD62L^-$, right) as shown in **A**. **C**, **D** Lymphocytes in the lung were concurrently analyzed and displayed in the same manner as in **A**, **B**. **E**–**G** Secondary immune response in Themis cKO mice. LCMV Arm-infected mice at 80 dpi were rechallenged with LCMV clone 13 and analyzed at Day 5 post rechallenge. **E** Viral titer in the organs of mice as indicated. **F** Summary of the proportion (left) and number (right) of LCMV-specific tetramer-positive $CD8^+$ T cells in the spleen. **G** The expression of IFNγ and TNF in GP33-41^+ cells in the spleen. Representative FACS plots (left) and summary of data (right) are shown. Data are representative of two independent experiments. Each symbol represents an individual mouse. *P* values were calculated by Student's *t* test (ns, nonsignificant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$)

response in Themis cKO mice and WT mice (as determined by virus titer).

Themis mediates PD-1 expression and its signaling in effector $CD8^+$ T cells

Our above results that Themis-deficient effector $CD8^+$ T cells generated more cytokines on a per-cell basis suggested that Themis may act as a negative regulator of effector $CD8^+$ T cells. Two potential mechanisms may account for this phenotype, either enhanced T-cell activation or, alternatively, impaired T-cell inhibition. To test these two possibilities, we adopted an in vitro TCR stimulation system in which we stimulated P14 TCR transgenic T cells with a dendritic cell line constitutively expressing the GP33-41 peptide (Fig. S8A). The P14 TCR recognizes the LCMV GP33-41 peptide presented by H2-D^b [41]. This approach allowed us to examine T-cell activation status at the very early stage in an antigen-specific manner, which is impractical to test in vivo. We first examined several cell surface markers indicative of T-cell activation and found that the expression of CD69, CD25, and CD44 showed various degrees of reduction or delay in P14 cKO cells at the beginning several hours but eventually reached a similar amount as that in P14 WT cells at 24 h post-stimulation (Fig. S8B). For CD62L, there were comparable kinetics of initial downregulation and later re-expression in both

types of P14 cells (Fig. S8B). These results demonstrated that P14 cKO cells were not hyperactivated compared to P14 WT cells, indicating that Themis was unlikely to be a negative regulator of T-cell activation. We next speculated that Themis might be involved in PD-1-orchestrated T-cell inhibition because Themis is associated with SHP-1 and SHP-2, two phosphatases that bind PD-1 and mediate its signaling [42]. We found that the upregulation of PD-1 was apparent in P14 WT cells at 5 h post-stimulation and escalated to a higher level at 24 h (Fig. S8C). However, the expression of PD-1 in P14 cKO cells exhibited a delay and reduction (Fig. S8C). This result implied that cKO $CD8^+$ T cells might be under less inhibition than their WT counterparts and supported the notion that Themis is involved in promoting T-cell inhibition.

The above in vitro findings, together with the report that PD-1 is upregulated on effector $CD8^+$ T cells upon LCMV Arm infection [43], prompted us to further examine the expression of PD-1 on P14 cKO cells in vivo. For this purpose, we cotransferred an equal number of P14 WT and P14 cKO cells into congenically marked recipient mice and subsequently infected these mice with LCMV Arm (Fig. 7A) [43]. Consistent with our in vitro findings, at 5 dpi, we found that the expression of PD-1 was severely reduced in P14 cKO cells, whereas the expression of CD25 and CD44 was comparable between P14 cKO and P14 WT cells (Fig. 7B). Notably, we also found that the proportion of P14 cKO cells was significantly lower than that of their WT counterparts (Fig. 7C, left). To understand the cause of the impaired expansion of P14 cKO cells, we compared the cell turnover rate and found that proliferation of P14 cKO cells was decreased (Fig. 7C, middle), while the death of P14 cKO cells was increased (Fig. 7C, right). We wondered whether the above findings were restricted to LCMV Arm infection. To answer this question, we infected another batch of recipient mice cotransferred with P14 cells but with a recombinant *Listeria monocytogenes* bacterial strain expressing the LCMV GP33-41 peptide. Under these conditions, the upregulation of PD-1 was much milder in both cell types than that seen in LCMV Arm infection, although P14 cKO cells still exhibited less expression of PD-1 than their WT counterparts (Fig. S9A). Similarly, the expansion rate of both cell types was reduced compared to the rate observed in LCMV Arm infection. Nonetheless, the proportional disadvantage of P14 cKO cells remained the same (Fig. S9B).

Although the above P14 cell cotransfer approach is widely used in the LCMV infection model due to its convenience of singular clone type and minimization of environmental influence, it lacks the heterogeneity of an endogenous TCR repertoire. Recently, a study revealed that GP33-41 tetramer-positive cells are comprised of an estimated hundreds of T-cell clones of varied TCR avidity [44], and this clonal heterogeneity led to diverse differentiation trajectories even though all the cells recognized the same LCMV GP33-41 epitope [44, 45]. We thus wondered whether our findings in P14 cells could be recapitulated in polyclonal GP33-41 tetramer-positive T cells. We found that at 5 dpi, the expression of PD-1 was decreased in GP33-41-positive T cells in cKO mice (Fig. 7D); however, it became comparable to that in their WT counterparts at 8 dpi (Fig. 7E). This result not only demonstrated the plasticity of PD-1 expression on GP33-41-positive endogenous polyclonal $CD8^+$ T cells but also prompted us to speculate whether Themis may be involved in PD-1 signaling as an additional mechanism of action. We reasoned this possibility because we previously identified an interaction between Themis and PD-1 in mouse thymocytes (Fig. 7F), where both Themis and PD-1 are highly expressed [46]. However, we could not obtain consistent results when using primary LCMV-specific T cells after viral infection due to the scarcity of cells. Enlightened by the interaction between Themis and PD-1, we further asked whether the abrogation of Themis may disrupt the association between PD-1 and its downstream effectors SHP-1 and SHP-2 and impair SHP phosphatase activity. To test this hypothesis, we sorted WT and cKO $CD8^+$ T cells from LCMV Arm-infected mice at 8 dpi and examined the phosphorylation of SHP-1 and SHP-2. We found that the phosphorylation of SHP-2, and to a lesser degree that of SHP-1, was severely impaired in Themis cKO $CD8^+$ T cells (Fig. 7G), suggesting decreased phosphatase activity. Collectively, these results suggested that Themis may function in a nonmutually exclusive way to mediate PD-1 expression as well as its downstream signaling, which can account for the enhanced cytokine production observed in Themis-deficient effector $CD8^+$ T cells during LCMV Arm infection.

DISCUSSION

In this study, using the LCMV Arm infection model, we showed that Themis-deficiency conferred $CD8^+$ T cells with some aspects of increased functionality, such as preferential differentiation into effector cells of the SLECs subset at the cost of MPECs, and enhanced production of effector cytokines both in primary immune response as well as in the memory stage. Mechanistically, we propose that Themis exerts these functions by mediating PD-1 expression and its downstream signaling in effector $CD8^+$ T cells, two nonmutually exclusive mechanisms.

Orchestrated TCR and cytokine signaling is a prerequisite for naive $CD8^+$ T cells to properly differentiate into effector cells [47]. While TCR signals generally determine the on and off status of T-cell differentiation, cytokine signals can tune the magnitude of T-cell differentiation. One striking finding in our present study is that, in both primary and memory stages, Themis-deficient virus-specific $CD8^+$ T cells consistently exhibited elevated effector function at the single-cell level but expanded poorly at the population level. Several factors may contribute to this asymmetric phenomenon. First, the homeostasis defect in Themis-deficient $CD8^+$ T cells may skew their TCR repertoire and provide a less than usual frequency of LCMV-specific T cells at the steady state [19]. Second, the impaired responsiveness of Themis-deficient $CD8^+$ T cells to IL-2 and IL-15 stimulation may further restrict their proliferation and survival capability [20]. Third, Themis-deficient $CD8^+$ T cells preferentially differentiate into SLECs and hence suffer from poor survival capability. Last, Themis seems to act as a suppressor of TCR signaling in mature T cells, and the disruption of Themis bolsters TCR signaling in mature $CD8^+$ T cells, consequently promoting their differentiation. Intriguingly, when we dynamically compared the behavior of Themis cKO $CD8^+$ T cells to their WT counterparts, such as the quantity of LCMV-specific T cells (Fig. 2F, G) and their cytokine production (Fig. S3, and Fig. 3), the inhibitory role of Themis only manifested at the late stage of LCMV Arm infection (8 dpi). This stage is, however, the exact time at which effector $CD8^+$ T-cell function needs to be curbed. In contrast, a stimulatory role of Themis is more likely in the early stage of infection to promote naive $CD8^+$ T-cell activation (up to 5 dpi), similar to its role in T-cell homeostasis.

Although the idea that Themis suppresses the function of mature $CD8^+$ T cells is consistent with our previous study in thymocytes [13], this view is still under debate, at least for thymocytes [16, 17]. Currently, there are two directly contrasting models of whether Themis positively or negatively regulates TCR signaling in thymocytes. Indeed, the dispute is mainly regarding how Themis affects the activity of SHP-1 [48]. A recent study tried to reconcile this discrepancy and found that the critical event to distinguish the above two models is the phosphorylation status of Themis [49]. Consistent with our previous report [6], this study showed that TCR activation rapidly induced Themis phosphorylation. However, they further discovered that phosphorylated Themis can act as a "priming substrate" to bind SHP-1 and escalate its basal level of phosphatase activity to nearly full strength, ensuring a downregulation of TCR signaling. In contrast,

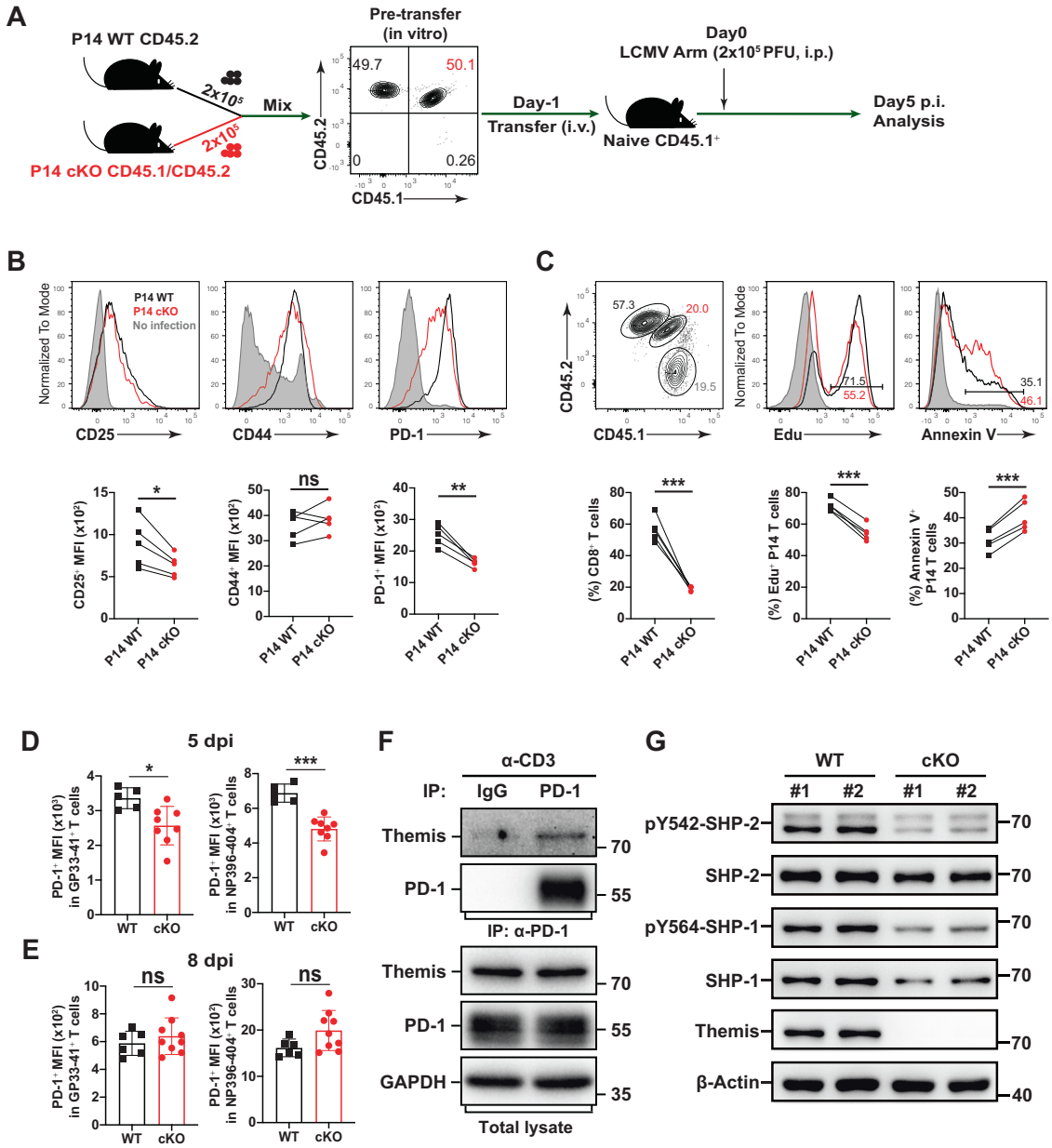

Fig. 7 Themis mediates PD-1 expression and signaling in effector CD8$^+$ T cells. **A** Schematic presentation of the experimental setup. **B** Expression of the indicated markers on cotransferred P14 WT and cKO cells as indicated. For all columns, representative FACS plots (top) and summary of data (bottom) are shown. **C** Cellular turnover of transferred P14 cells. Left column: the proportion of transferred P14 WT and cKO cells. Middle column: the proliferation status of P14 cells measured by EdU incorporation assay. Right column: Cell death of P14 cells measured by Annexin V staining. For all columns, representative FACS plots (top) and summary of data (bottom) are shown. **D**, **E** Expression of PD-1 on LCMV-specific tetramer-positive polyclonal CD8$^+$ T cells at 5 and 8 dpi. **F** Themis and PD-1 interaction. Endogenous coimmunoprecipitation of PD-1 and Themis was performed in anti-CD3-stimulated thymocytes. GAPDH blot indicates the input of each sample. **G** Immunoblot analyses of the phosphorylation of SHP-1 and SHP-2 in primary CD8$^+$ T cells after sorting from mice at 8 dpi. **A**–**E** Data are representative of two experiments, and each symbol indicates an individual mouse. **F** Representative of two experiments. **G** Two pairs of WT and cKO mice were analyzed and are representative of two experiments. *P* values were calculated by Student's *t* test (ns, nonsignificant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$)

cytokine signaling in CD8$^+$ T cells does not elicit Themis phosphorylation [49]. Thus, this study revealed both phosphorylation-dependent and phosphorylation-independent roles of Themis in controlling T-cell maturation and expansion [49]. Interestingly, a very recent study also reported a dual role of Themis in CD4$^+$ T cells [50]. The authors found a stimulatory function for Themis on type 1 cytokine responses in effector CD4$^+$ T cells in an experimental autoimmune encephalomyelitis (EAE) model. In contrast, in in vitro studies, Themis exerted an opposite effect on naive CD4$^+$ T cells by inhibiting TCR-mediated signals that lead to Th1 cell responses [50]. Nonetheless, the elevated cytokine (IFNγ) production in CD4$^+$ T cells observed in vitro in the above study mirrored our findings in CD8$^+$ T cells during LCMV Arm infection.

An effective immune response apparently required the combination of single cell level functionality and bulk cell level force of numbers, with the result being a balance of the two. Given the contrasting configurations in these two branches

between Themis cKO and WT mice, it was remarkable that both strains of mice could resolve LCMV Arm infection effectively. The implication from this finding is that there is probably a large space for $CD8^+$ T cells to deal with an acute infection. It will be of great interest to see how Themis cKO mice respond to a chronic viral infection, where the virus exists persistently rather than being rapidly cleared.

When we formally speculated that Themis may play an inhibitory role in effector $CD8^+$ T cells, one conundrum is that we did not observe an enhanced T-cell activation phenotype in the absence of Themis. Instead, we observed mildly attenuated T-cell activation in Themis-deficient $CD8^+$ T cells (Fig. S8B). Therefore, we considered the alternative possibility that Themis may promote T-cell inhibition and eventually discovered that the abrogation of Themis would "break the brake" either by inhibiting PD-1 expression (Fig. 7B) or interfering with its downstream signaling (Fig. 7G). Although downregulation of PD-1 expression as a mechanism of breaking T-cell inhibition has been previously reported [51], our finding that Themis bound to and mediated PD-1 downstream signaling revealed another novel mechanism. This mechanism seemed to coordinate well with our findings that Themis exerts its inhibitory function at the late stage of infection. Remarkably, delineation of this mechanism originated from the observed discrepancy in PD-1 expression between endogenous GP33-41-positive polyclonal T cells and the transferred P14 TCR transgenic monoclonal T cells. We believe that with the rapid advances in single-cell TCR sequencing (scTCR-seq) and scRNA-seq technology, more discoveries will be made in dissecting the clonal heterogeneity of a given antigen-specific T-cell population (as defined by single tetramer staining) and its biological importance [44, 45]. In our view, it is probably high time to adopt multiple TCR clones when addressing a fundamental question. Heavily relying on a single TCR clone could disguise some important discoveries, as demonstrated in our study here.

The enigmatic role of Themis and the complexity of Themis' own regulation undoubtedly emphasize the significance of this molecule in T cells and warrant more in-depth studies. We believe that it will be critical to define more context-dependent functions of Themis in the future, which is particularly relevant when considering translating these basic findings into clinical applications.

MATERIALS AND METHODS

Mice

Themis$^{flox/flox}$-dLck-Cre mice were generated and kindly provided by Dr. Nicholas Gascoigne at the National University of Singapore [19]. P14 TCR transgenic mice were originally obtained from Dr. Charles Surh at the Scripps Research Institute. All mice were maintained under specific pathogen-free conditions at the animal center at Xiamen University. For all experiments, mice of sex-matched littermates at 8–12 weeks old were used, and the experiments were approved by the Institutional Animal Care and Use Committee of Xiamen University.

Viral infection and titers

LCMV Armstrong (Arm) and LCMV Clone13 (C13) strains were propagated in BHK-21 cells and titrated on Vero cells. For infection, 2×10^5 plaque-forming units (PFUs) of LCMV Arm were injected intraperitoneally into each mouse. A total of 2×10^6 plaque-forming units (PFUs) of LCMV C13 were injected intravenously into each mouse. Viral titers were determined in mouse serum by qPCR assay as previously described [24]. Briefly, a standard curve was generated by serial dilution of a viral stock with a predetermined titer in PFU/ml. RNA was extracted from standard samples and tested samples, and the titer of tested samples was converted to PFU/ml according to the standard curve.

Histology

Organs were harvested from infected mice, placed in PBS-buffered formalin, and then blocked in paraffin. Five-micron tissue sections were stained with hematoxylin and eosin. Microscopic images were captured on a Motic VM1 Microscopic digital slice scanning system.

In vivo cytotoxicity assay

To prepare target cells, one part of splenocytes was incubated with a low concentration of 0.25 μM CFSE and pulsed with LCMV GP33-41 peptide. Equal portions of the same splenocytes were incubated with a high concentration of 5 μM CFSE but without peptide. Both splenocyte fractions were mixed at a 1:1 ratio and cotransferred into LCMV Arm-infected mice at Day 8 post-infection. Three hours later, target cell elimination was determined by flow cytometry analysis of the CFSE profile.

In vivo cell proliferation assay

At 5 dpi, infected mice were i.p. injected with 2 μg of EdU (Beyotime, China) 4 h before analysis. To measure cell proliferation, cells were intracellularly stained with anti-EdU antibody and analyzed by flow cytometry. EdU staining was performed using an EdU kit (BeyoClick EdU Cell Proliferation Kit, Beyotime, China) following the manufacturer's instructions.

In vitro T-cell activation assay

To activate T cells, naive P14 cells were purified and cocultured with a DC2.4 cell line engineered to express the LCMV GP33-41 epitope at 37 °C.

Flow cytometry

For tetramer staining, cells were incubated with MHC-I tetramers H-2D^b/GP33–41, H-2D^b/GP276–286, or H-2D^b/NP396–404 before any additional surface staining. All tetramers were made in the lab. For cell surface staining, cells were incubated with an antibody cocktail at 4 °C for 30 min. The antibody cocktail was prepared by diluting antibodies in FACS buffer (PBS supplemented with 0.5% BSA and 0.01% azide). In the cocktail, the following antibodies were used: KLRG1 (2F1), IFNγ (XMG1.2), TNF (MP6-XT22), granzyme B (NGZB), and CD45.2 (104) from eBioscience and CD8α (53-6.7), CD44 (IM7), CD127 (A7R34), PD-1 (29 F.1A12), CD90.1 (OX-7), and CD90.2 (53-2.1) from Biolegend. For intracellular cytokine staining, cells were first restimulated in vitro with GP33-41 peptide (5 μg/ml) for 5 h in the presence of GolgiPlug (1 μL/10^6 cells/ml) and then fixed and permeabilized using the Cytofix/Cytoperm Kit (BD).

Immunoprecipitation and immunoblotting

For immunoprecipitation, mouse thymocytes were prepared from the thymus of niave mice and resuspended in lysis buffer. The cell lysate was centrifuged at 13,000 rpm for 10 min at 4 °C to generate a cleared supernatant, and protein A/G beads were added to the supernatant followed by rotation at 4 °C for 45 min. Anti-PD-1 antibody was added to the supernatant and incubated at 4 °C overnight with rotation. Then, protein A/G beads were added and rotated for 2 h at 4 °C. The beads were washed with immunoprecipitation buffer three times at 4 °C and then mixed with an equal volume of 2x SDS sample buffer for western blotting. For immunoblotting, primary $CD8^+$ T cells were FACS sorted from infected mice and subjected to analyses. The anti-PD-1 antibody was generated by Dr. Chenghao Huang at Xiamen University. For immunoblotting, the following antibodies were used: anti-SHP-1 (C14H6), anti-phospho-SHP-1 (Y564) (D11G5), anti-SHP-2 (D50F2) and anti-phospho-SHP-2 (Y542) (#3751 S) from Cell Signaling Technology; anti-Themis (EPR7353) from Abcam; and anti-beta actin (2D4H5) from Proteintech.

DATA AVAILABILITY

For flow cytometry analysis, antibody-stained cells were run on an LSR-Fortessa X-20 analyzer (BD). All flow cytometry data were analyzed using FlowJo v.10.7.1 software (TreeStar). Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). GraphPad Prism was used for statistical analysis to compare outcomes using a two-tailed unpaired Student's *t* test and two-tailed paired Student's *t* test for cotransfer experiments; $P > 0.05$ was considered not significant (ns); $^*P \le 0.05$; $^{**}P \le 0.01$; $^{***}P \le 0.001$ was considered statistically significant. All summarized data are shown in graphs with mean±sd.

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G.F., Q.L., and X.L.C. designed and supervised the study. J.T. X.J. J.L. and J.D. performed most experiments with help from others. C.L., W.L., and Q.L. provided critical reagents. N.R.J.G. and G.F. wrote the manuscript with input from all authors.

COMPETING INTERESTS

The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to Xiao Lei Chen, Qing Li, Nicholas R. J. Gascoigne or Guo Fu.