

# The kinase GLK controls autoimmunity and NF- $\kappa$ B signaling by activating the kinase PKC- $\theta$ in T cells

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Protein kinase C- $\theta$  (PKC- $\theta$ ) is required for activation of the transcription factor NF- $\kappa$ B induced by signaling via the T cell antigen receptor (TCR); however, the direct activator of PKC- $\theta$  is unknown. We report that the kinase GLK (MAP4K3) directly activated PKC- $\theta$  during TCR signaling. TCR signaling activated GLK by inducing its direct interaction with the upstream adaptor SLP-76. GLK-deficient mice had impaired immune responses and were resistant to experimental autoimmune encephalomyelitis. Consistent with that, people with systemic lupus erythematosus had considerable enhanced GLK expression and activation of PKC- $\theta$  and the kinase IKK in T cells, and the frequency of GLK-overexpressing T cells was directly correlated with disease severity. Thus, GLK is a direct activator of PKC- $\theta$ , and activation of GLK-PKC- $\theta$ -IKK could be used as new diagnostic biomarkers and therapeutic targets for systemic lupus erythematosus.

NF- $\kappa$ B is a major transcription factor that regulates genes encoding molecules responsible for cell survival, growth and proliferation. Engagement of the T cell antigen receptor (TCR) induces NF- $\kappa$ B activation, which is involved in host defense against infection and the development of inflammation, cancer and autoimmunity. Protein kinase C- $\theta$  (PKC- $\theta$ ) has a critical role in the activation of inhibitor of NF- $\kappa$ B (I $\kappa$ B) kinase (IKK) and NF- $\kappa$ B and functions of T cells<sup>1</sup>. The adaptor SLP-76 is required for PKC- $\theta$  activation after TCR stimulation<sup>2,3</sup>. However, the signal transduction from SLP-76 to PKC- $\theta$  and the direct kinase that activates PKC- $\theta$  have remained unclear. Thus, the most critical issue in TCR-induced NF- $\kappa$ B activation is the identification of the pivotal link between SLP-76 and PKC- $\theta$ .

PKC- $\theta$  activation requires phosphorylation at Thr538 (ref. 4). The kinase PDK1 interacts with PKC- $\theta$ , and phosphorylation of PKC- $\theta$  at Thr538 is defective in PDK1-deficient T cells<sup>5</sup>. Thus, PDK1 has been proposed to directly phosphorylate PKC- $\theta$  at Thr538, although no clear evidence exists that PDK1 directly phosphorylates PKC- $\theta$  *in vitro*. Furthermore, the observation that PDK1 can be activated only by the coreceptor CD28, and hence not by TCR signaling<sup>5</sup>, further rules out the possibility that PDK1 is the direct kinase for PKC- $\theta$  activation induced by TCR signaling. Thus, the kinase that directly activates PKC- $\theta$  during T cell activation remains elusive.

GCK-like kinase (MAP4K3) is a member of the MAP4K family (mitogen-activated protein kinase kinase kinase kinase), which is a subfamily of Ste20-like serine-threonine kinases<sup>6</sup>. GLK contains a conserved N-terminal kinase domain, a conserved C-terminal citron homology domain and several proline-rich motifs in between<sup>6</sup>. Phosphorylation of the MAPK Jnk is induced by GLK via the MAPKs

MEKK1 and MKK4 (SEK1) in response to stress stimulation<sup>6</sup>. GLK also regulates cell growth by activating the kinase mTOR downstream effectors S6K1 and 4E-BP1 in epithelial cell lines after amino acid treatment<sup>7</sup>. However, the regulatory mechanism and physiological roles of GLK remain largely unknown. Because another member of the MAP4K family, HPK1 (MAP4K1), is a critical negative regulator of TCR signaling<sup>8</sup>, we examined the role of GLK in the TCR signaling cascade and found that GLK is the kinase that links signal transduction from SLP-76 to PKC- $\theta$ .

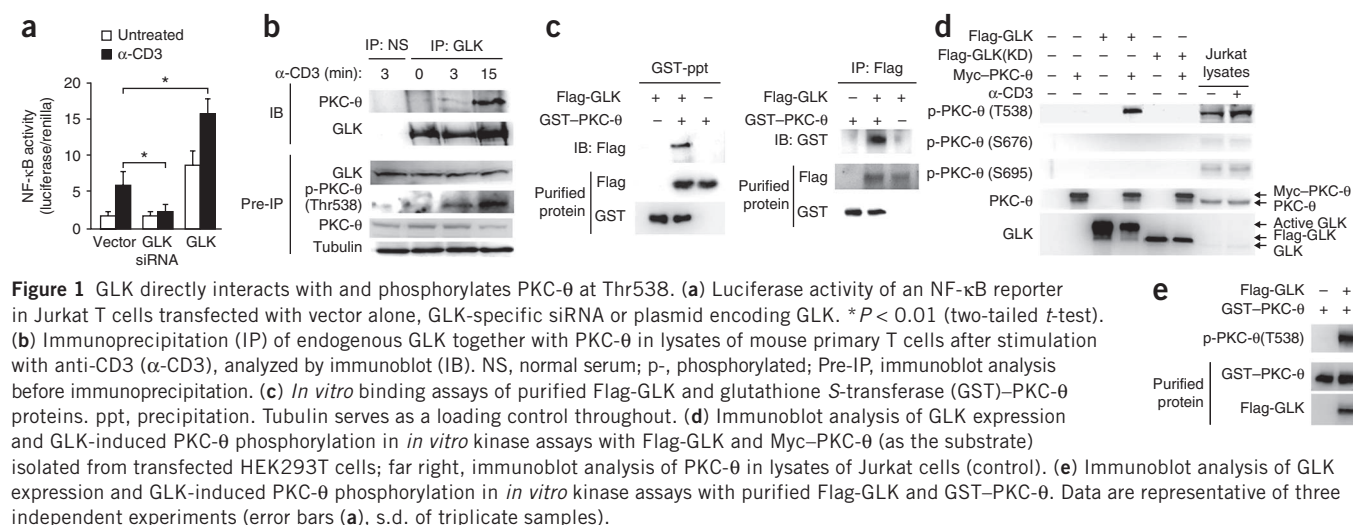
## RESULTS

### GLK directly phosphorylates and activates PKC- $\theta$

We studied the role of GLK in TCR signaling by GLK overexpression or knockdown of GLK via small interfering RNA (siRNA) in Jurkat T cells followed by stimulation with antibody to the coreceptor CD3 (anti-CD3; **Fig. 1a** and **Supplementary Fig. 1**). Anti-CD3-induced NF- $\kappa$ B activity and IKK phosphorylation, but not activation of the MAPK Erk or mTOR, in Jurkat T cells were suppressed by GLK-specific siRNA or a mutant of GLK without kinase activity ('kinase-dead' GLK) and were enhanced by GLK overexpression (**Fig. 1a** and **Supplementary Fig. 2a,b**). Moreover, GLK kinase activity was induced by TCR signaling at 1 min and peaked at 30 min after stimulation with anti-CD3 (**Supplementary Fig. 2c**). These findings suggested that GLK is involved in TCR-induced NF- $\kappa$ B activation. Because SLP-76 regulates both Erk and IKK pathways, SLP-76 was unlikely to be the target of GLK. We thus examined the effect of GLK on the activation of PKC- $\theta$ , a critical regulator upstream of IKK-NF- $\kappa$ B and downstream of SLP-76 in TCR signaling. PKC- $\theta$  phosphorylation was regulated by GLK

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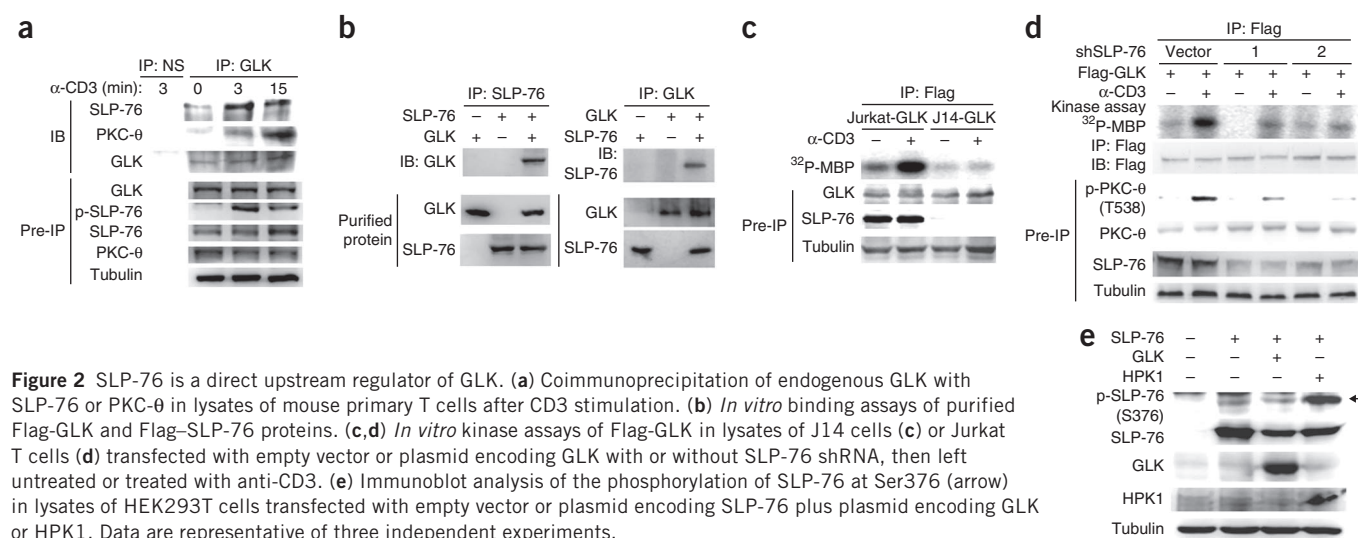
(Supplementary Fig. 2d); therefore, we examined whether GLK activated the IKK-NF-κB pathway by targeting PKC-θ. Knockdown of PKC-θ via siRNA abolished GLK-induced NF-κB activation after stimulation with anti-CD3 (Supplementary Fig. 2e), which indicated that GLK targets PKC-θ to induce NF-κB activation.

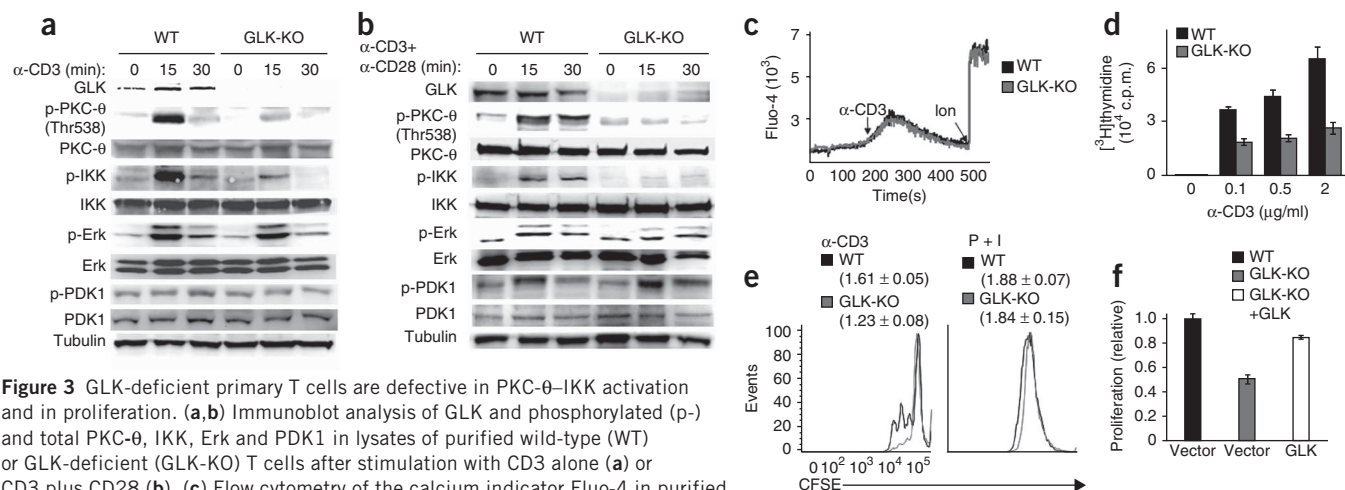
To determine if GLK directly phosphorylates and activates PKC-θ during T cell signaling, we investigated whether GLK interacted with PKC-θ during TCR signaling by coimmunoprecipitation assays. Stimulation with anti-CD3 induced interaction between endogenous GLK and PKC-θ in mouse primary T cells, and the GLK-PKC-θ interaction was concomitant with PKC-θ activation (Fig. 1b). We obtained similar results with HEK293T human embryonic kidney cells, Jurkat human T cells and EL4 mouse thymoma cells (Supplementary Fig. 3a–c). We further showed direct interaction between GLK and PKC-θ *in vitro* with binding assays of purified GLK and PKC-θ proteins (Fig. 1c). To determine the sites of phosphorylation of PKC-θ by GLK, we examined PKC-θ phosphorylation at Thr538, Ser676 and Ser695 (ref. 4), which are the three main phosphorylation sites of PKC-θ. Phosphorylation at Thr538 is the most critical site for activation of PKC-θ and subsequent activation of NF-κB. *In vitro* kinase assays with immunoprecipitated Flag-tagged GLK and Myc-tagged PKC-θ showed that GLK phosphorylated PKC-θ at Thr538 but not at Ser676 or Ser695 (Fig. 1d).

In *in vitro* kinase assays with purified GLK and PKC-θ proteins, PKC-θ was directly phosphorylated at Thr538 by GLK (Fig. 1e). Together our results indicated that GLK directly interacts with PKC-θ and phosphorylates PKC-θ at Thr538 during TCR signaling.

### SLP-76 is a direct upstream regulator of GLK

SLP-76 is a critical scaffold protein in T cells and is required for TCR-induced activation of PKC-θ-IKK<sup>2</sup>. We thus sought to determine whether SLP-76 is a direct upstream regulator of GLK in TCR signaling. Stimulation with anti-CD3 induced interaction between endogenous GLK and SLP-76 in mouse primary T cells (Fig. 2a). The SLP-76-GLK interaction preceded both the GLK-PKC-θ interaction and PKC-θ activation. We also obtained similar results with Jurkat and HEK293T cells (Supplementary Fig. 4a–c). Moreover, the interaction between SLP-76 and GLK was mediated by tyrosine phosphorylation (Supplementary Fig. 4d). *In vitro* binding assays with purified GLK and SLP-76 showed a direct interaction between these two proteins (Fig. 2b). Next we studied whether SLP-76 is required for GLK activation. The kinase activity of GLK induced by stimulation of CD3 was abolished in SLP-76-deficient J14 human T cells (Fig. 2c) and Jurkat cells transfected with short hairpin RNA (shRNA) targeting SLP-76 (Fig. 2d). A published report has shown that the guanine





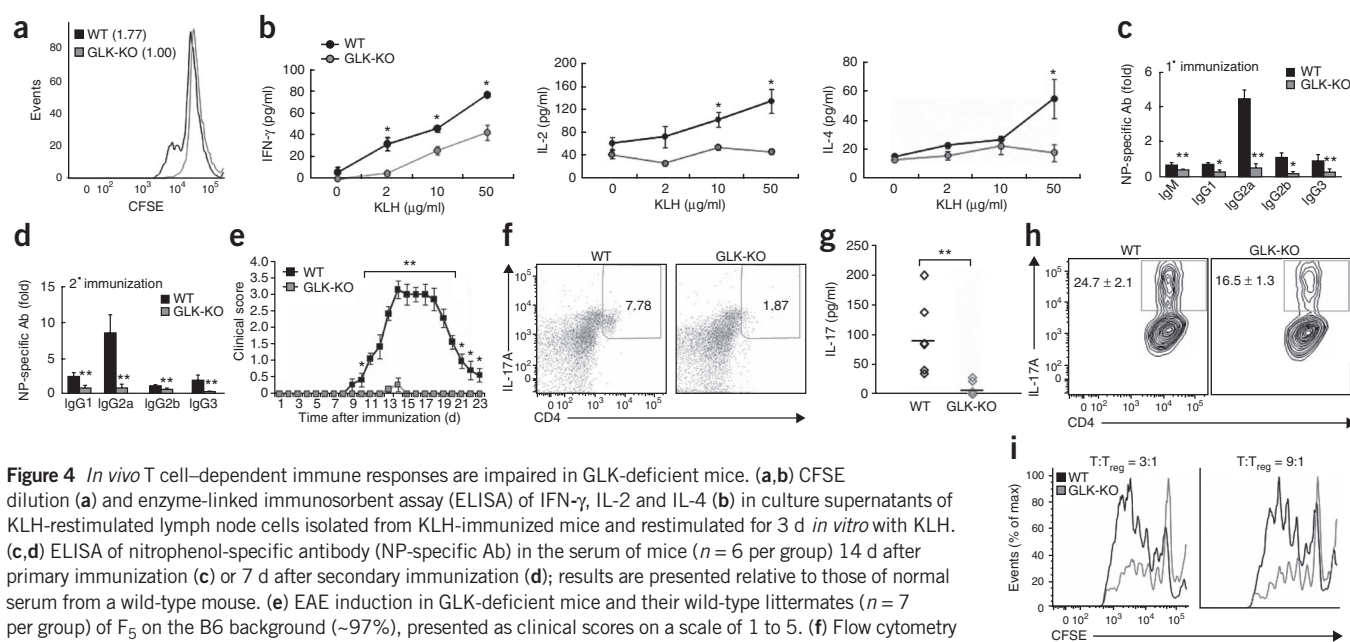
**Figure 3** GLK-deficient primary T cells are defective in PKC- $\theta$ -IKK activation and in proliferation. (a,b) Immunoblot analysis of GLK and phosphorylated (p-) or total PKC- $\theta$ , IKK, Erk and PDK1 in lysates of purified wild-type (WT) or GLK-deficient (GLK-KO) T cells after stimulation with CD3 alone (a) or CD3 plus CD28 (b). (c) Flow cytometry of the calcium indicator Fluo-4 in purified mouse T cells stimulated with anti-CD3 and then with ionomycin (Ion). (d,e) [ $^3$ H]thymidine incorporation (d) and CFSE dilution (e) by wild-type or GLK-deficient CD3 $^+$  T cells treated for 72 h with anti-CD3 or PMA plus ionomycin (P + I). (f) [ $^3$ H]thymidine incorporation in wild-type and GLK-deficient T cells transfected with empty vector (encoding green fluorescent protein alone) or vector encoding GLK tagged with green fluorescent protein. Data are representative of three independent experiments (error bars (d,f), s.e.m.).

nucleotide-exchange factor Vav1 is associated with SLP-76 and is involved in PKC- $\theta$  translocation<sup>9</sup>, which suggests that Vav1 controls PKC- $\theta$  activation. Thus, we studied whether Vav1 regulated GLK activation. However, TCR-induced activation of GLK was not affected by shRNA-mediated knockdown of Vav1 (Supplementary Fig. 5). These data ruled out the possibility of involvement of Vav1 in the regulation of GLK kinase activation in TCR signaling. As another MAP4K, HPK1, is also activated by SLP-76 and in turn negatively regulates SLP-76 activation by inducing SLP-76 phosphorylation at Ser 376 (refs. 8,10), we investigated whether GLK was involved in the negative feedback regulation of SLP-76. The phosphorylation of SLP-76 at Ser376 was induced only by HPK1 and thus not by GLK (Fig. 2e),

which suggested that GLK does not provide feedback to negatively regulate SLP-76. These findings indicate that SLP-76 directly interacts with and activates GLK during TCR signaling.

#### GLK controls T cell-mediated immune responses *in vivo*

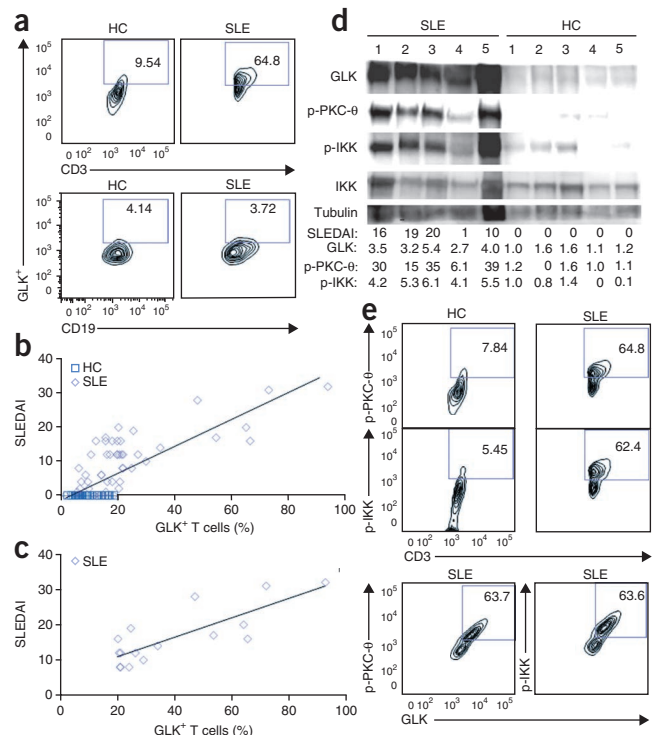
To study the role of GLK *in vivo*, we generated GLK-deficient mice (Supplementary Fig. 6a,b) and found that T cell development was normal in these mice (Supplementary Fig. 6c–e). We then studied the effect of GLK deficiency on TCR signaling in primary T cells. Consistent with our observations reported above (Fig. 1), the phosphorylation of both PKC- $\theta$  and IKK was abolished in GLK-deficient T cells after stimulation with anti-CD3 (Fig. 3a) or with anti-CD3



**Figure 4** *In vivo* T cell-dependent immune responses are impaired in GLK-deficient mice. (a,b) CFSE dilution (a) and enzyme-linked immunosorbent assay (ELISA) of IFN- $\gamma$ , IL-2 and IL-4 (b) in culture supernatants of KLH-restimulated lymph node cells isolated from KLH-immunized mice and restimulated for 3 d *in vitro* with KLH. (c,d) ELISA of nitrophenol-specific antibody (NP-specific Ab) in the serum of mice ( $n = 6$  per group) 14 d after primary immunization (c) or 7 d after secondary immunization (d); results are presented relative to those of normal serum from a wild-type mouse. (e) EAE induction in GLK-deficient mice and their wild-type littermates ( $n = 7$  per group) of F<sub>5</sub> on the B6 background (~97%), presented as clinical scores on a scale of 1 to 5. (f) Flow cytometry of infiltrating TH17 cells (CD45 gated) from the brains and spinal cords of myelin oligodendrocyte glycoprotein (MOG)-immunized mice on day 14. (g) ELISA of IL-17 in serum from MOG-immunized mice ( $n = 6$  per group). (h) Flow cytometry of IL-17A-producing CD4 $^+$  T cells among *in vitro*-differentiated TH17 cells. (i) Suppression of CFSE-labeled CD3 $^+$  T cells by wild-type or GLK-deficient T<sub>reg</sub> cells, presented as CFSE dilution in responding T cells cultured at a ratio of 3:1 (left) or 9:1 (right) with T<sub>reg</sub> cells plus anti-CD3-coated beads. Numbers in or adjacent to outlined areas indicate percent cells in each throughout. \* $P < 0.05$  and \*\* $P < 0.001$  (two-tailed  $t$ -test). Data are from two (a,c,d,g,i) or three (b,e,f,h) independent experiments (mean  $\pm$  s.e.m.).



**Figure 5** GLK expression and phosphorylation of PKC- $\theta$  at Thr538 are induced in T cells from people with SLE. **(a)** Flow cytometry analysis of GLK-expressing (GLK<sup>+</sup>) lymphocytes from PBLs of people with SLE ( $n = 49$ ) and unaffected controls (HC;  $n = 35$ ); the results from one person with SLE (SLEDAI, 20) are presented here. **(b)** Positive correlation and significant regression between SLEDAI and the frequency of GLK-expressing T cells from all people with SLE (Pearson correlation coefficient  $r = 0.773$ ; simple linear regression,  $y = -0.886 + 0.3901x$ ; regression correlation coefficient, adjusted  $r^2 = 0.597$ ,  $P = 1.08 \times 10^{-17}$ ). **(c)** Correlation and significant regression between SLEDAI and the frequency of GLK<sup>+</sup> T cells from people with SLE with high frequency of GLK<sup>+</sup> cells ( $\geq 21\%$ ;  $n = 16$ ; Pearson correlation coefficient  $r = 0.807$ ; simple linear regression,  $y = 5.2085 + 0.2757x$ ; regression correlation coefficient, adjusted  $r^2 = 0.626$ ,  $P = 0.000159$ ). **(d)** Immunoblot analysis of GLK and phosphorylated PKC- $\theta$  and IKK in lysates of PBLs from people with SLE ( $n = 5$ ; randomly sampled) and healthy controls ( $n = 5$ ). Below lanes, SLEDAI and densitometry results, presented relative to tubulin. **(e)** Flow cytometry analysis of phosphorylated PKC- $\theta$  or IKK among (CD3-gated) PBLs of with a person with SLE and a healthy control **(a)**. Data are representative of 35 **(a)**, at least three **(d)** or at least 16 **(e)** independent experiments.



plus anti-CD28 (**Fig. 3b**). In contrast, activation of Erk and PDK1 was unaffected by GLK deficiency (**Fig. 3b**). Phosphorylation of the tyrosine kinase (Lck), the membrane adaptor Lat and phospholipase C- $\gamma 1$ , as well as  $\text{Ca}^{2+}$  signaling, were also unaffected (**Fig. 3c** and **Supplementary Fig. 6f**). As NF- $\kappa\text{B}$  signaling regulates T cell proliferation, we also studied the effect of GLK on T cell proliferation by analysis of [ $^3\text{H}$ ]thymidine incorporation and dilution of the cystolic dye CFSE (which dilutes progressively with division). GLK deficiency significantly blocked T cell proliferation induced by stimulation with anti-CD3 ( $P = 0.013$ ) but not proliferation induced by stimulation with phorbol 12-myristate 13-acetate (PMA) plus ionomycin ( $P = 0.621$ ; **Fig. 3d,e** and **Supplementary Fig. 6g**). Because PMA and ionomycin bypass proximal TCR signaling, this result suggested that GLK activity is induced by the TCR-proximal signaling complex. Furthermore, the diminished proliferation of GLK-deficient T cells was 'rescued' by ectopically expressed GLK (**Fig. 3f**). These data show that GLK is important for T cell proliferation.

We next investigated whether GLK has an important role in T cell-mediated immune responses *in vivo*. We immunized wild-type and GLK-deficient mice with keyhole limpet hemocyanin (KLH), a T cell-dependent antigen, using alum as an adjuvant. T cells from KLH-immunized GLK-deficient mice showed hypoproliferation after KLH restimulation (**Fig. 4a**). Moreover, the amounts of cytokines, including interferon- $\gamma$  (IFN- $\gamma$ ), interleukin 2 (IL-2) and interleukin 4 (IL-4), were also significantly lower in KLH-restimulated splenic T cells from GLK-deficient mice (**Fig. 4b**), which suggested impaired *in vivo* T cell activation. The production of antigen-specific antibodies in the serum after primary and secondary immunization was also much lower in GLK-deficient mice (**Fig. 4c,d**). These results indicate that GLK is required for mounting immune responses and antibody production.

### GLK-deficient mice are resistant to autoimmunity

Experimental autoimmune encephalomyelitis (EAE) is mediated mainly by CD4<sup>+</sup> lymphocytes of the interleukin 17 (IL-17)-producing helper T subset ( $\text{T}_\text{H}17$  cells)<sup>11</sup>. PKC- $\theta$  deficiency results in ameliorated EAE and  $\text{T}_\text{H}17$  responses<sup>12,13</sup>. Because GLK activates PKC- $\theta$ , we studied the role of GLK in  $\text{T}_\text{H}17$ -mediated autoimmune diseases with the EAE model. GLK-deficient mice barely showed any symptoms, whereas their wild-type littermates developed severe EAE (**Fig. 4e**). The frequency of

$\text{T}_\text{H}17$  cells in the infiltrating lymphocytes in the brains and spinal cords of immunized mice at day 14 was significantly lower in GLK-deficient mice than in wild-type mice (**Fig. 4f**). In addition, the titer of IL-17 in serum was also significantly lower in GLK-deficient mice than in wild-type mice (**Fig. 4g**). To investigate whether the defective  $\text{T}_\text{H}17$  response in GLK-deficient mice was a T cell-intrinsic effect, we determined the *in vitro*  $\text{T}_\text{H}17$ -differentiation potential of GLK-deficient naive T cells. Consistent with the results obtained above, the *in vitro* differentiation of  $\text{T}_\text{H}17$  cells, as well as of type 1 helper T cells ( $\text{T}_\text{H}1$ ) and type 2 helper T cells ( $\text{T}_\text{H}2$ ), was diminished by GLK deficiency (**Fig. 4h** and **Supplementary Fig. 7**). *In vitro* differentiation of regulatory T cells ( $\text{T}_\text{reg}$  cells) was not affected by GLK deficiency (**Supplementary Fig. 7**); however, GLK-deficient  $\text{T}_\text{reg}$  cells had greater suppressive activity *in vitro* (**Fig. 4i**). These results indicated that GLK deficiency *in vivo* protects mice from the development of  $\text{T}_\text{H}17$ -mediated autoimmune diseases. Defective TCR signaling and  $\text{T}_\text{H}17$  differentiation, as well as enhanced  $\text{T}_\text{reg}$  cell function, of GLK-deficient T cells may have critical roles in the resistance of GLK-deficient mice.

### GLK-induced PKC- $\theta$ activation in human autoimmune disease

$\text{T}_\text{H}17$ -mediated inflammation also has an important role in the human autoimmune disease systemic lupus erythematosus (SLE)<sup>14</sup>. Because autoimmune induction and  $\text{T}_\text{H}17$  responses were attenuated in GLK-deficient mice and because GLK activates the PKC- $\theta$ -IKK pathway, we studied whether the GLK-PKC- $\theta$ -IKK-IL-17 cascade is involved in human SLE. We examined GLK expression and PKC- $\theta$ -IKK activation in peripheral blood leukocytes (PBLs) isolated from 49 people with SLE and 35 paired healthy controls (**Supplementary Fig. 8a**). Flow cytometry analysis showed that the frequency of GLK-expressing T cells, but not of B cells, from freshly isolated PBL samples from people with SLE was much greater (**Fig. 5a**). The frequency of GLK-expressing T cells correlated with the SLE disease activity index (SLEDAI; **Fig. 5b**; Pearson correlation coefficient  $r = 0.773$ ). Notably, one third (16 of 49) of the people with SLE and a high percentage of T cells with GLK expression ( $\geq 21\%$

**Table 1 Correlation of SLEDAI and GLK<sup>+</sup> T cell frequency in SLE**

Subsets of people with SLE and GLK <sup>+</sup> T cells	SLEDAI (mean)	Pearson correlation coefficient (r)
Total	9.6	0.773
≥21%	16.4	0.807
<21%	6.9	0.451

For people with SLE and low GLK<sup>+</sup> T cells (<21%), the adjusted regression correlation coefficient is  $r^2 = 0.177$ , with  $P = 0.008503$ .

GLK<sup>+</sup> cells) showed higher correlation with SLEDAI ( $r = 0.807$ ; **Fig. 5c** and **Table 1**) than did people with SLE and GLK expression in the normal range ( $r = 0.451$ ; **Table 1**). GLK protein expression was also higher in people with SLE (**Fig. 5d**). These results suggest that GLK overexpression is involved in SLE pathogenesis in 30% of people affected.

We next investigated whether GLK overexpression in T cells from people with SLE-induced PKC- $\theta$ -IKK activation. The frequency of T cells with phosphorylated PKC- $\theta$  or IKK was greater, and these cells costained with GLK expression in T cells from people with SLE (**Fig. 5e**). Similarly, immunoblot analysis showed induction of PKC- $\theta$ -IKK activation in people with SLE (**Fig. 5d**). These data suggested that GLK activates PKC- $\theta$  in T cells from people with SLE. The titer of IL-17, but not of tumor necrosis factor or IL-6, was enhanced in serum from people with SLE (**Supplementary Fig. 8b**). These results suggested that GLK overexpression in T cells drives autoimmune pathogenesis in people with SLE. Thus, GLK acts an important positive regulator of autoimmune diseases by directly activating PKC- $\theta$  in T cells.

## DISCUSSION

A key finding of our study was the identification of GLK as a kinase that directly phosphorylated PKC- $\theta$  at Thr538 during TCR signaling. PKC- $\theta$  phosphorylation at residue Thr538 in the activation loop is essential to NF- $\kappa$ B activation in T cells<sup>4</sup>. GLK-induced phosphorylation of PKC- $\theta$ -IKK was independent of PDK1 activation and of CD28 costimulation, which suggests that GLK functions independently of PDK1 activation. These data indicate that GLK is the direct upstream kinase for PKC- $\theta$  in TCR signaling.

The results of *in vitro* T cell differentiation assays further indicated that GLK has an intrinsic and positive role in helper T cell differentiation. The mechanism by which GLK regulates helper T cell differentiation remains unknown. However, *in vitro* T<sub>H</sub>1, T<sub>H</sub>2 and T<sub>H</sub>17 differentiation were all diminished by GLK deficiency, which suggests that GLK may regulate helper T cell differentiation through a common mechanism (for example, by enhancing TCR signaling or IL-2 production) instead of by regulating different cytokine signaling pathways for different helper T cell differentiation pathways.

Our studies of the *in vivo* roles of GLK showed that GLK was required for optimal T cell immune responses and for antibody production. The lower abundance of T cell-secreted cytokines, such as IL-2, IFN- $\gamma$  (a T<sub>H</sub>1 cytokine) and IL-4 (a T<sub>H</sub>2 cytokine), as well as the lower production of antigen-specific antibodies after primary and secondary immunization of GLK-deficient mice are similar to deficiencies reported for PKC- $\theta$ -deficient T cells<sup>13,15,16</sup>. The resistance of GLK-deficient mice to EAE induction and diminished T<sub>H</sub>17 responses are also consistent with the phenotype of PKC- $\theta$ -deficient mice<sup>12,13</sup>. The number of thymic natural T<sub>reg</sub> cells was unaffected in GLK-deficient mice. However, T<sub>reg</sub> cell numbers are impaired in mice deficient in PKC- $\theta$ , the scaffolding protein CARMA1 or the adaptor Bcl-10 or mice with mutant IKK2 because of impaired PKC- $\theta$ -NF- $\kappa$ B signaling<sup>17–21</sup>. PKC- $\theta$  can be activated by multiple signaling pathways such as CD28, CD4, CD8, integrin  $\alpha_L\beta_2$  (LFA-1) or endoplasmic

reticulum stress<sup>22,23</sup>. It remains unclear how PKC- $\theta$ -NF- $\kappa$ B signaling is activated during T<sub>reg</sub> cell development. Our data indicates that GLK activates PKC- $\theta$  by phosphorylating the Thr538 residue but not other known phosphorylation sites (such as Ser676 or Ser695). GLK is probably not the only PKC- $\theta$ -activating kinase. Therefore, it is likely that PKC- $\theta$ -CARMA1-Bcl-10-IKK2 can still be activated by other kinases in T<sub>reg</sub> cells, leading to normal numbers of T<sub>reg</sub> cells in GLK-deficient mice. Although T<sub>reg</sub> cell numbers were unaffected in GLK-deficient mice, T<sub>reg</sub> cell activity was enhanced by GLK deficiency. PKC- $\theta$  has a negative role in T<sub>reg</sub> cell-mediated suppression<sup>24</sup>. The role of GLK in regulating T<sub>H</sub>1, T<sub>H</sub>2, T<sub>H</sub>17 and T<sub>reg</sub> cells was essentially consistent with that of PKC- $\theta$ , which supports the idea that GLK is probably the sole activator of PKC- $\theta$  in TCR signaling.

In this study we also reported that GLK expression was induced in peripheral blood T cells of people with SLE and was positively correlated with SLE disease severity. T cells with GLK overexpression isolated from people with SLE also showed more PKC- $\theta$ -IKK activation, which suggested that activation of the GLK-PKC- $\theta$ -IKK pathway controls SLE pathogenesis. This is the first report to our knowledge of GLK overexpression and PKC- $\theta$ -IKK hyperactivation in T cells of people with SLE. The higher concentration of serum IL-17 in people with SLE is consistent with published reports<sup>14</sup>. The correlation between GLK and SLEDAI ( $r = 0.773$ ) is much higher than the correlation between serum IL-17 and SLEDAI (Pearson correlation coefficient  $r = 0.41$ ), and is significant ( $P = 1.08 \times 10^{-17}$ ). Together our findings indicate that GLK has an important role in autoimmune pathogenesis and could serve as a new diagnostic biomarker for the progression of SLE; in addition, the inhibition of GLK and its downstream PKC- $\theta$ -IKK signaling may offer new therapeutic strategies for SLE.

## METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureimmunology/>.

Note: Supplementary information is available on the Nature Immunology website.

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## AUTHOR CONTRIBUTIONS

H.-C.C. designed and did experiments, analyzed and interpreted data and wrote the manuscript; J.-L.L. and D.-Y.C. provided samples from affected people, analyzed clinical data and coordinated clinical research; C.-Y.Y., J.-P.L. and P.-E.L. did experiments; Y.-M.C. provided samples from affected people and analyzed clinical data; C.-Y.H. assisted in generation of the GLK-deficient mice; X.W. assisted in the experimental design and manuscript writing; and T.-H.T. conceived of the study, supervised experiments and composed the manuscript.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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## ONLINE METHODS

**GLK-deficient mice.** A 129 mouse embryonic stem cell clone with knockout GLK (RRO270) from the European Conditional Mouse Mutagenesis Project was injected into blastocysts from the mouse line C57BL/6 to generate chimeric mice at the Transgenic Mouse Model Core, National Research Program for Genomic Medicine of Taiwan. All animal experiments were according to protocol approved by the Institutional Animal Care and Use Committee at the National Health Research Institutes, Taiwan.

**People with SLE and healthy controls.** Forty-nine people diagnosed with SLE based on American College of Rheumatology criteria were studied. All people with SLE were referred to the Division of Allergy, Immunology and Rheumatology at Taichung Veterans General Hospital (Taichung, Taiwan). Thirty-five healthy people were enrolled as controls. The study was approved by the institutional review board of the Taichung Veterans General Hospital, and written informed consent was obtained from all subjects.

**Antibodies, plasmids and purified proteins.** Anti-mouse CD3 $\epsilon$  (145-2C11) and anti-human CD3 $\epsilon$  (OKT3) were purified from mouse ascites by protein A-Sepharose chromatography. Anti-GLK<sup>6</sup>, anti-HPK1, antibody to Erk phosphorylated at Thr202 and Tyr204, anti-Erk, antibody to SLP-76 phosphorylated at Ser376 and anti-SLP-76 were generated by immunization of rabbits with the appropriate peptide. Antibody to PKC- $\theta$  phosphorylated at Thr538 (07-885) and to PKC- $\theta$  phosphorylated at Ser695 (07-885) were from Upstate Biotechnology. Anti-PKC- $\theta$  (C-19), antibody to PKC- $\theta$  phosphorylated at Ser676 (sc-33024), anti-Vav1 (D-7), anti-Vav2 (E-12) and anti Vav3 (K-19) were from Santa Cruz Biotechnology. Anti-Flag (M2), anti-Myc (9E10), anti-hemagglutinin (HA-7) and anti-tubulin (T4026) were from Sigma. Antibody to IKK phosphorylated at Ser181 (2681S), PDK1 phosphorylated at Ser241 (3061), anti-IKK (10AG2) and anti-PDK1 (3062) were from Cell Signaling. Antibody to PKC- $\theta$  phosphorylated at Thr538 (19/PKC) for intracellular staining was from BD Pharmingen. Antibody to phosphorylated mTOR (ab63552) and anti-mTOR (ab2723) were from Abcam.

Expression plasmids for GLK, the GLK kinase-dead mutant and Flag-SLP-76 have been described<sup>6,8</sup>. Myc-tagged PKC- $\theta$ , hemagglutinin-tagged GLK or green fluorescent protein-tagged GLK were constructed by subcloning of the appropriate cDNA into vector pCMV6-AC-Myc, pCMV6-AC-hemagglutinin or pCMV6-AC-GFP, respectively (OriGene Technologies). The shRNA plasmids were established by the National RNAi Core Facility (Taiwan). NF- $\kappa$ B reporter plasmid (pNF- $\kappa$ B-luciferase) and normalized plasmid (pRL-TK) were from Promega.

For *in vitro* binding assays, purified GLK or SLP-76 was isolated from HEK293T cells transfected with Flag-GLK or Flag-SLP-76, respectively, followed by Flag-peptide elution. Purified recombinant GST-PKC- $\theta$  expressed from baculovirus in Sf9 insect cells was from SignalChem.

**Transient transfection and T cell activation.** For transient transfection assays, cells were transfected with the Neon Transfection System (Invitrogen). Specific settings for cell lines or primary cells were as follows: 1,420 V for a duration of 30 ms and 1 pulse for Jurkat T and J14 cell lines; 1,080 V for a duration of 50 ms and 1 pulse for EL4 cells; and 2,000 V for a duration of 20 ms and 2 pulses for primary T cells. For induction of T cell activation, J-TAg human T cells and EL4 cells were stimulated with 5  $\mu$ g/ml of anti-CD3 for the appropriate time at 37 °C. For induction of primary T cell activation,  $3 \times 10^6$  purified T cells were stimulated with 3  $\mu$ g/ml biotin-conjugated anti-CD3 (500A2; eBioscience) plus 3  $\mu$ g/ml of streptavidin (Sigma).

***In vitro* kinase assays.** Flag-GLK was immunoprecipitated from unstimulated or anti-CD3-stimulated Jurkat or J14T cell lysates (80  $\mu$ g) with anti-Flag

agarose beads. Samples immunoprecipitated with anti-Flag (M2; Sigma) were either washed three times with lysis buffer and one time with kinase buffer or were subjected to elution of Flag-peptide for purification of Flag-GLK. Anti-Flag-GLK beads or purified Flag-GLK were incubated for 40 min at 25 °C with 500  $\mu$ M nonradioactive adenosine triphosphate (ATP), 4  $\mu$ g myelin basic protein and with or without 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP in 35  $\mu$ l kinase buffer. Samples were separated by SDS-PAGE. Phosphorylation of the substrate was detected by immunoblot analysis or the incorporation of isotope-labeled phosphate into the substrate, which was then quantified by a Typhoon scanner (GE).

**Flow cytometry analysis.** Cells were stimulated for 2 h with PMA (phorbol 12-myristate 13-acetate) plus ionomycin and treated for another 2 h with GolgiStop (BD Biosciences). Cells were collected, washed at 4 °C with PBS and stained for 30 min on ice with the appropriate antibodies. For clinical sample analysis, PBLs were immediately treated with GolgiStop without any other stimulation and then stained with antibodies to surface markers at 25 °C. For intracellular staining, PBLs were permeabilized for 2 h in 200  $\mu$ l Cytofix/Cytoperm buffer (BD Biosciences) and washed with Perm/Wash buffer and then incubated for 2 h in antibodies (1:50 dilution). The following antibodies were used for staining (all from BioLegend): allophycocyanin-anti-mouse CD3 (145-2C11), fluorescein isothiocyanate-anti-mouse CD3 (145-2C11), phycoerythrin-anti-Foxp3 (150D), fluorescein isothiocyanate-anti-IFN- $\gamma$  (XMG1.2), phycoerythrin-anti-IL-4 (11B11) and phycoerythrin-anti-IL-17A (TC11-18H10.1). Pacific blue-anti-MCDA (RM4-5), phycoerythrin-indotricarbocyanine-anti-human CD3 (SK7) and phycoerythrin-anti-human CD19 (SJ25C1) were from BD Pharmingen. Data were collected with a FACSCanto II (BD Biosciences) and were analyzed with FlowJo software.

***In vivo* T cell-mediated immune responses and induction of EAE.** Mice used in each experiment were 6- to 10-week-old sex-matched littermates. The production of antigen-specific (KLH) antibodies and T<sub>H</sub>1 and T<sub>H</sub>2 cytokines from immunized mice was measured as described<sup>8</sup>. EAE was induced as described<sup>8</sup>.

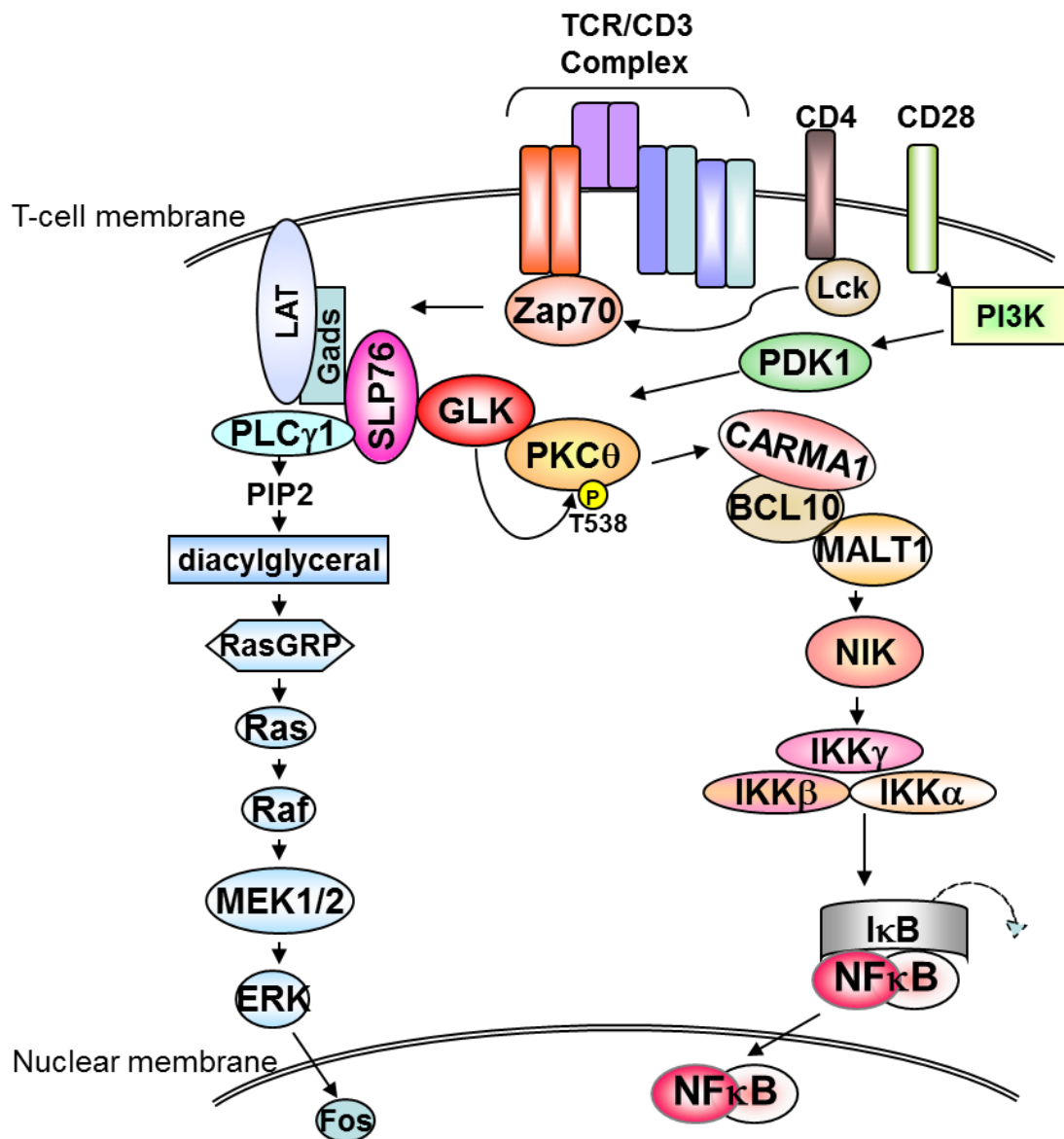
***In vitro* T cell-differentiation assays.** CD4<sup>+</sup>CD25<sup>-</sup> cells were purified from the lymph nodes of mice. Cells ( $2.5 \times 10^5$ ) were cultured in 500  $\mu$ l medium in 48-well plates coated with anti-CD3 (2  $\mu$ g/ml) and anti-CD28 (3  $\mu$ g/ml). For T<sub>reg</sub> cell differentiation, cells were cultured in medium containing 10 ng/ml of IL-2, 10 ng/ml of TGF- $\beta$  (transforming growth factor beta), 2.5  $\mu$ g/ml of anti-IL-4 and 2.5  $\mu$ g/ml of anti-IFN- $\gamma$ . For T<sub>H</sub>17 differentiation, cells were cultured in medium containing 20 ng/ml of IL-6, 5 ng/ml of TGF- $\beta$ , 50 ng/ml of interleukin 23 (IL-23), 5  $\mu$ g/ml of anti-IL-4 and 5  $\mu$ g/ml of anti-IFN- $\gamma$ . For T<sub>H</sub>1 differentiation, cells were cultured in medium containing 5 ng/ml of IL-12 and 1  $\mu$ g/ml of anti-IL-4. For T<sub>H</sub>2 differentiation, cells were cultured in medium containing 10 ng/ml of IL-4 and 1  $\mu$ g/ml of anti-IFN- $\gamma$ .

***In vitro* suppression assays.** Mouse CD4<sup>+</sup> T cells were negatively selected from the spleen and lymph nodes of mice. In the second round of purification, CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells were isolated from the CD4<sup>+</sup> T cells using magnetically coupled antibodies to mouse CD25. T<sub>reg</sub> cells were added to CD3<sup>+</sup> T cells (at a final concentration of  $2 \times 10^5$  cells per 500  $\mu$ l) and then stimulated for 72 with anti-CD3h.

**Statistical methods.** P values were determined by Student's *t*-tests. For analysis of data derived from clinical samples, Pearson correlation (*r*) coefficients were used. Simple linear regression for flow cytometry data of clinical samples was used to show a statistically significant correlation between the frequency of GLK-expressing T cells and SLEDAI in people with SLE.

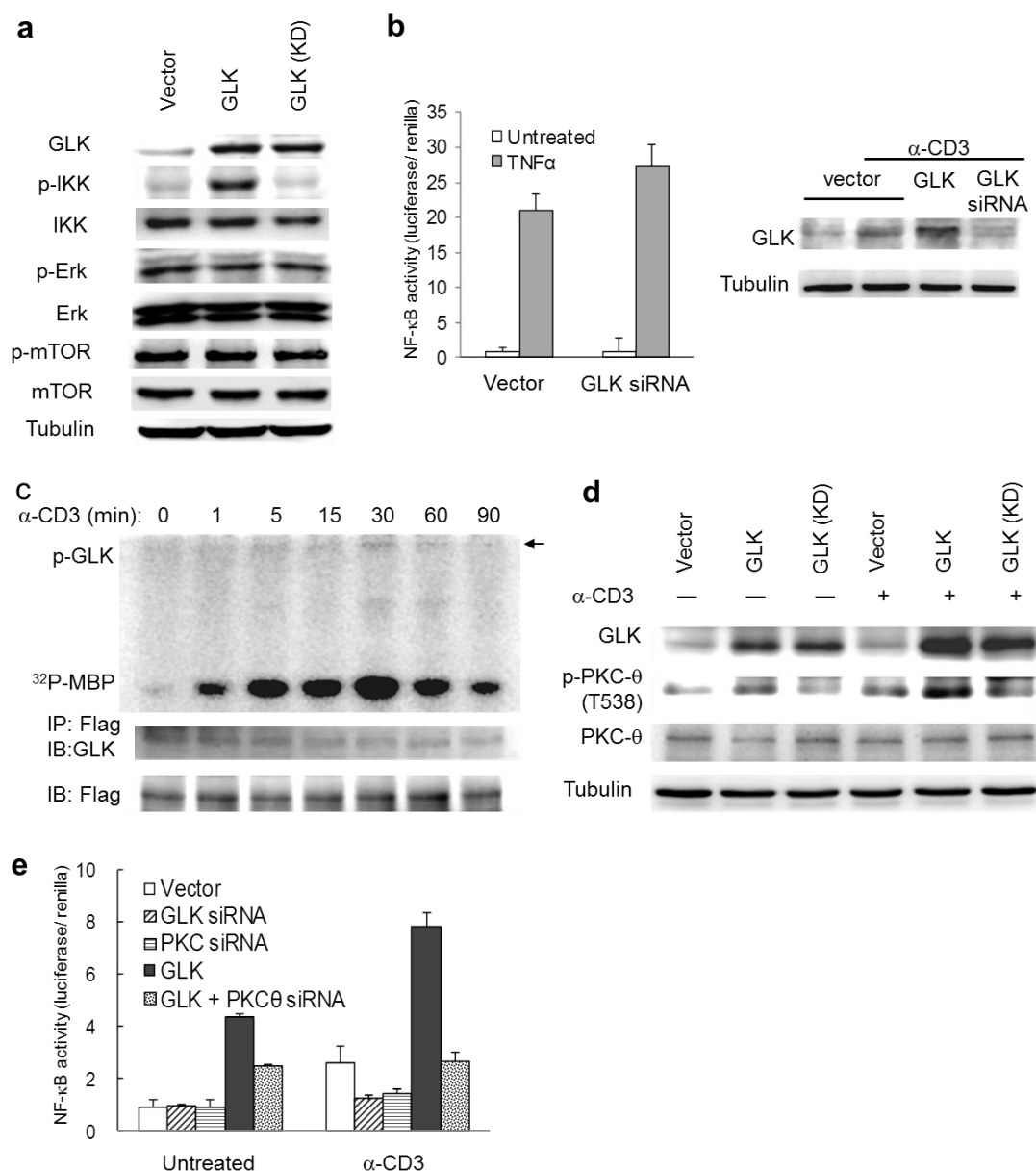


# Supplementary Figures:



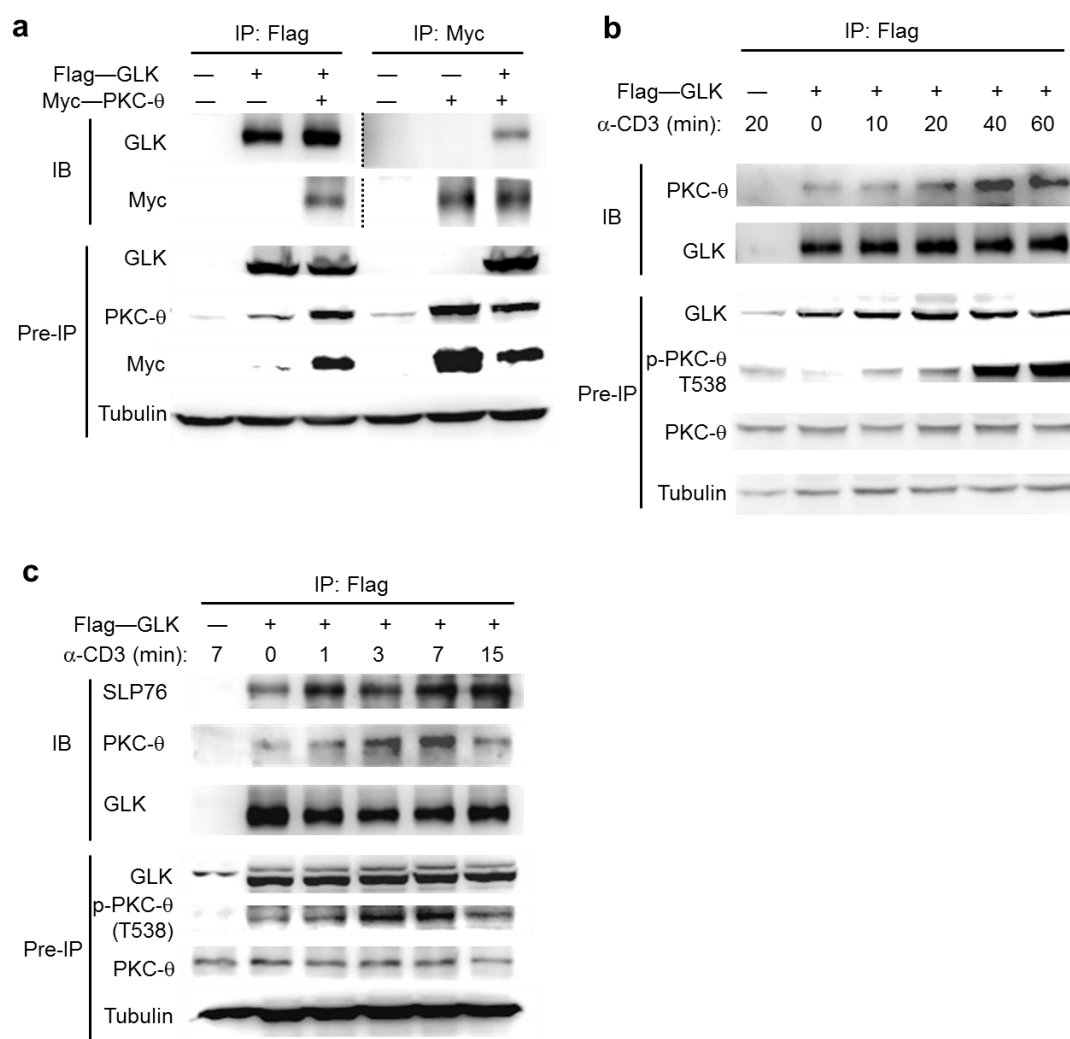
**Supplementary Figure 1. Diagram of GLK-induced PKC-θ/NF-κB activation during TCR signaling.** After TCR ligation, activated Lck is recruited to TCR complex and phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) of CD3, resulting in Zap70 recruitment and activation. Zap70 activation induces the assembly of the proximal SLP-76 signaling complex. SLP-76 directly interacts with GLK and is required for GLK kinase activation. The activated GLK directly interacts with and phosphorylates PKC-θ at T538, resulting in PKC-θ membrane translocation and kinase activation. The activated PKC-θ binds to and phosphorylates the signaling scaffold protein CARMA1, resulting in the activation of CARMA1 and the subsequent assembly of CARMA1-BCL10-MALT1 (CBM) complex. The CBM complex in turn induces the activation of the IKK/NF-κB signaling cascade.



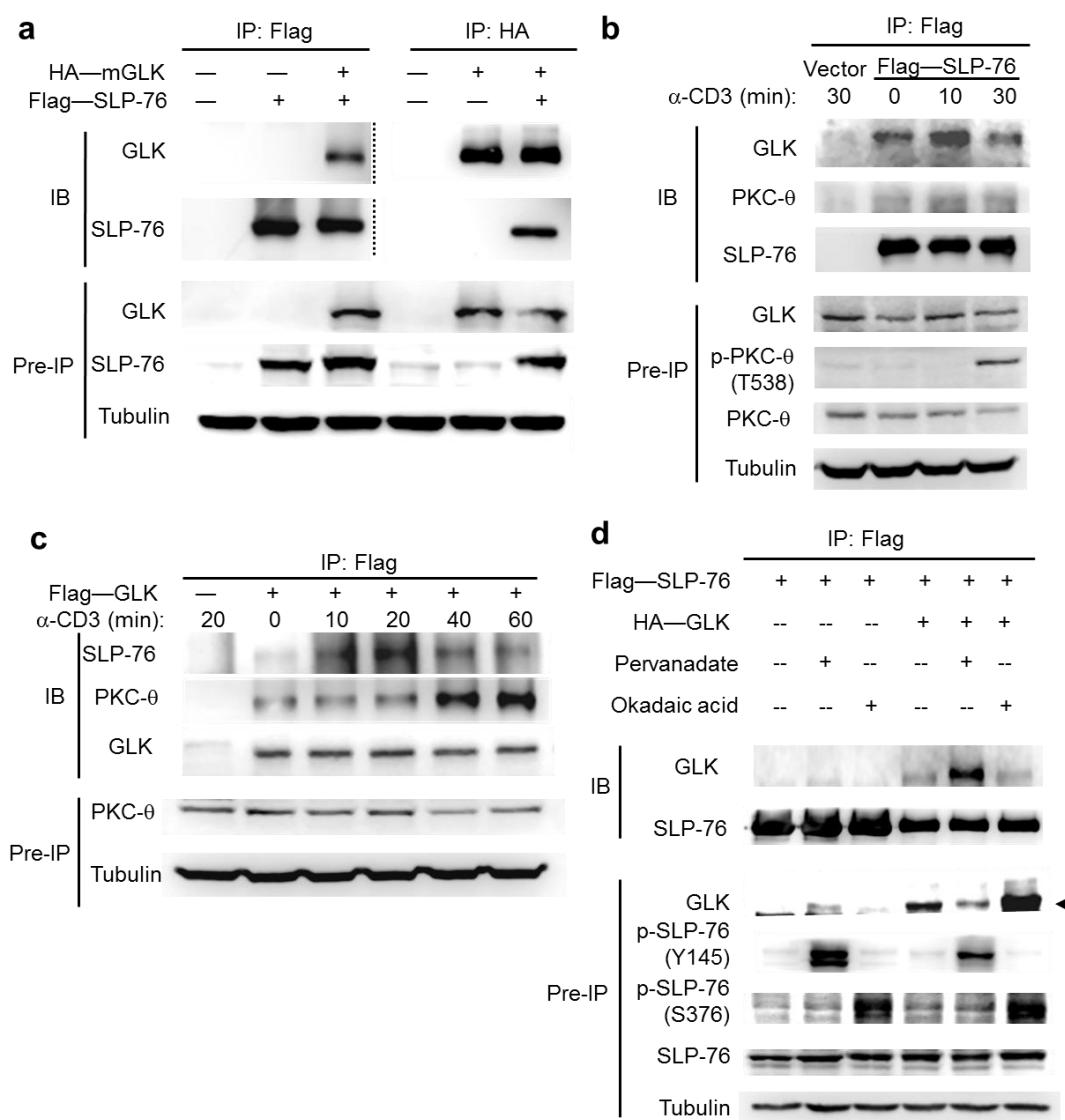


**Supplementary Figure 2. GLK induces NF- $\kappa$ B activation in T cells upon TCR stimulation.** (a) Immunoblot analyses of GLK, p-IKK, IKK, p-Erk, Erk, p-mTOR, mTOR and tubulin in the lysates of vector, GLK, or GLK(KD) mutant-expressing Jurkat T cells stimulated with anti-CD3 antibodies for 15 min. (b) NF- $\kappa$ B reporter assays of Jurkat cells transfected with empty vector or GLK siRNA following TNF- $\alpha$  stimulation (left). Immunoblot analyses of GLK and tubulin in lysates of Jurkat T cells transfected with empty vector, plasmid encoding GLK or GLK siRNA following anti-CD3 stimulation. (c) *In vitro* kinase assays of GLK isolated from Flag-GLK-expressing J-TAg T cells stimulated with anti-CD3 antibodies. Arrow, GLK autophosphorylation. IP, immunoprecipitation. IB, immunoblot. (d) Immunoblot

analyses of GLK, p-PKC- $\theta$ , PKC- $\theta$  and tubulin in lysates of J-TAg T cells transfected with plasmid encoding GLK or the GLK(KD) mutant following CD3 stimulation for 30 min. (e) NF- $\kappa$ B reporter assays of Jurkat T cells transfected with indicated empty vector, plasmid encoding GLK, GLK or PKC- $\theta$  siRNA alone, or plasmids encoding GLK plus PKC- $\theta$  siRNA. Cells were stimulated with or without anti-CD3 antibodies for 2 h. Data are representative of three independent experiments. \*,  $P$  value < 0.05; \*\*,  $P$  value < 0.001. Error bars in panels (b) and (e) are standard deviations (s.d.) of triplicate samples. Data are representative of at least three independent experiments.

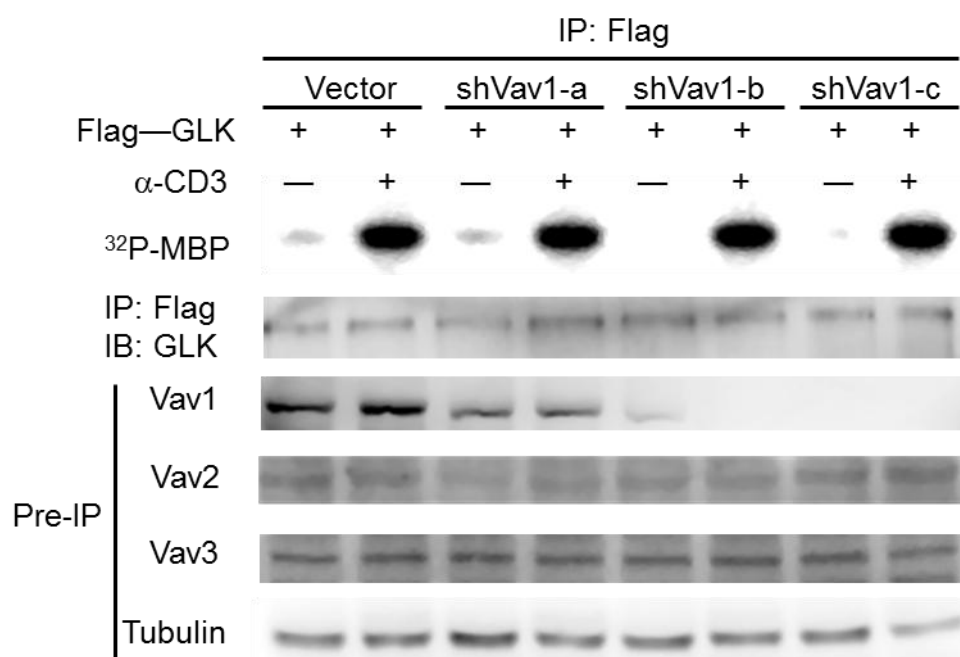


**Supplementary Figure 3. GLK interacts with PKC-θ.** (a) Co-immunoprecipitation (IP) and immunoblot (IB) analyses of GLK and PKC-θ in lysates of HEK293T cells transfected with empty vector or plasmid encoding GLK plus with or without plasmid encoding PKC-θ. (b,c) Co-immunoprecipitations of the Flag-GLK and endogenous PKC-θ in lysates of Flag-GLK-expressing J-Tag (b) or EL4 (c) T cells stimulated with 5 µg/ml anti-CD3 antibodies (upper panel). Immunoblot analyses of GLK, p-PKC-θ, PKC-θ and tubulin in pre-immunoprecipitation samples (lower panel). Data are representative of at least three independent experiments.



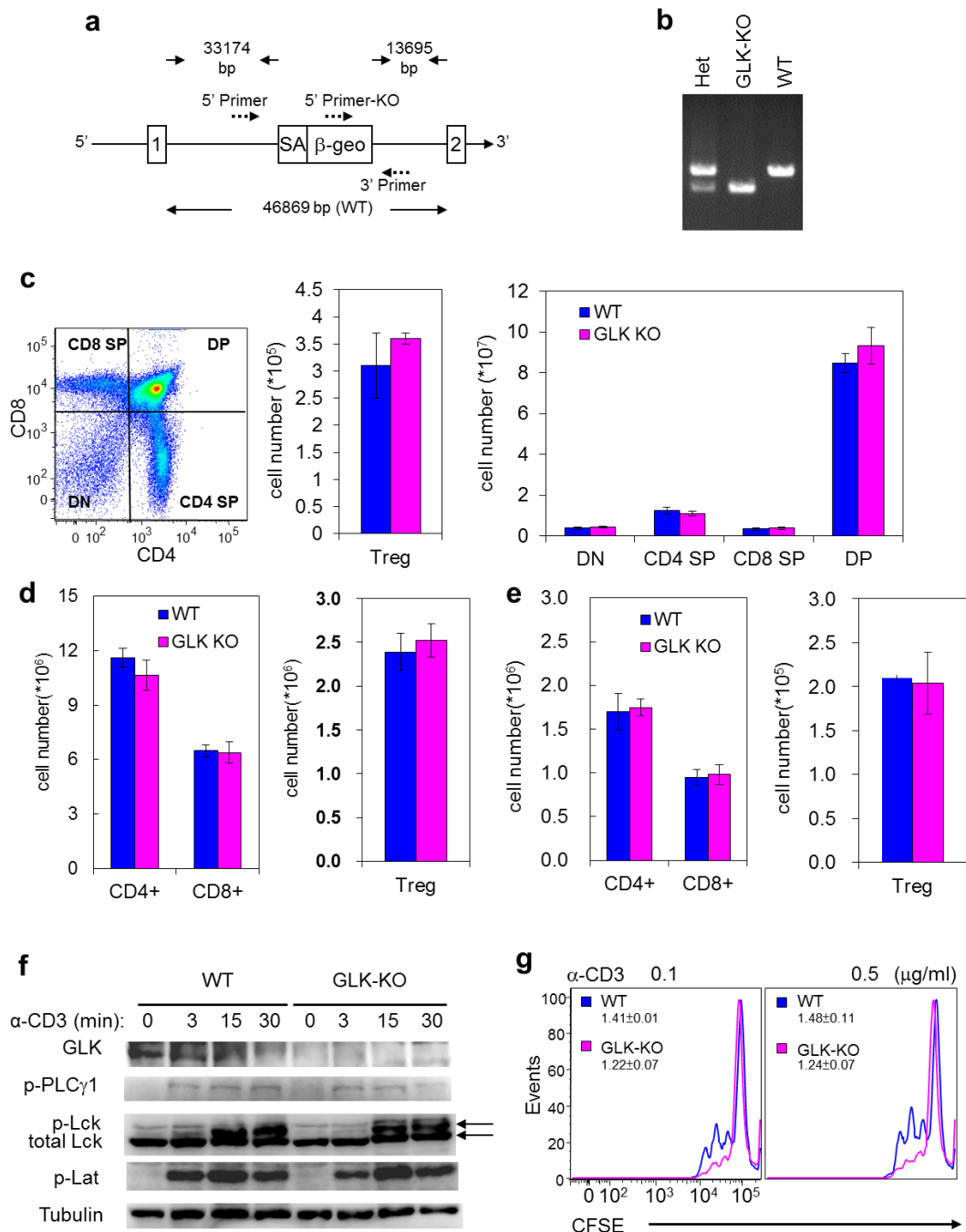
**Supplementary Figure 4. The interaction between GLK and SLP-76 is mediated through tyrosine phosphorylation.** (a) Co-immunoprecipitations (IP) and immunoblot (IB) analyses of GLK and SLP-76 in lysates of HEK293T cells transfected with empty vector or plasmid encoding SLP-76 plus with plasmid encoding GLK. (b) Co-immunoprecipitations of SLP-76 and GLK in lysates of Flag-SLP-76-overexpressed J-TAG T cells stimulated with anti-CD3 antibodies. (c) Co-immunoprecipitations of anti-CD3-induced SLP-76/GLK/PKC $\theta$  interaction in J-TAG T cells transfected with Flag-GLK. (d) Co-immunoprecipitations of GLK and SLP-76 in lysates of HEK293T cells transfected with plasmid encoding SLP-76 plus empty vector or plasmid encoding GLK. Cells were pre-treated with/without the tyrosine phosphatase inhibitor pervanadate or the serine/threonine phosphatase inhibitor okadaic acid. Data are representative of at least three independent experiments.





**Supplementary Figure 5. Vav1 is not required for TCR-induced GLK activation.**

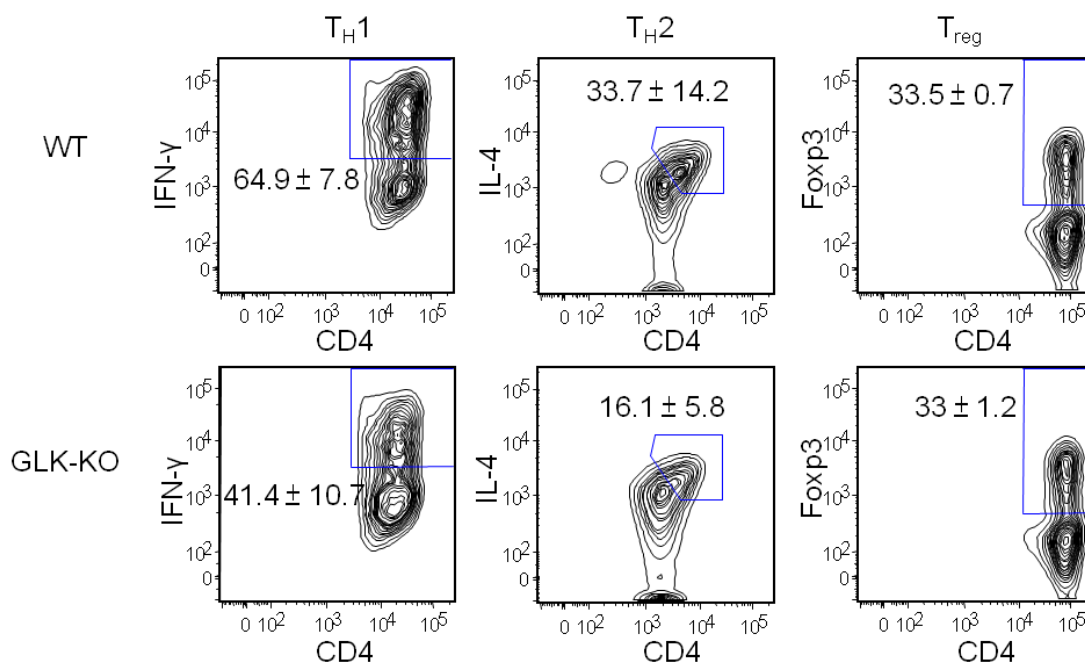
*In vitro* kinase assays of Flag-GLK isolated from the Vav1 shRNA-knocked down Jurkat T cells (shVav1 a-c) stimulated for 30 min with or without anti-CD3 antibodies. MBP was used as the substrate. Data are representative of at least three independent experiments.



**Supplementary Figure 6. T cell development is normal in GLK-deficient mice. (a)**

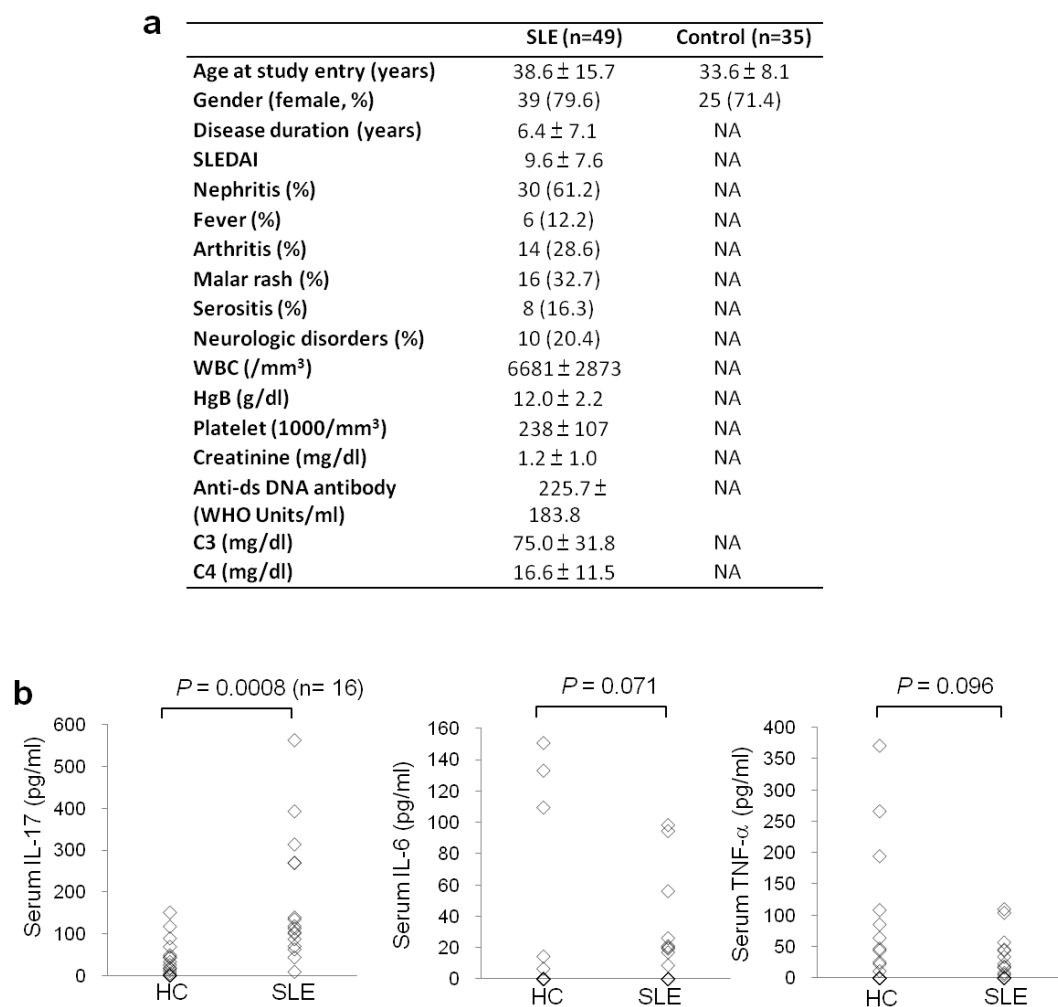
The structure of the gene-trap vector. β-geo, a fusion with β-galactosidase and neomycin phosphotransferase genes; SA, splicing acceptor; the box with numbers, the exon of GLK; dotted arrow, the primers for PCR. **(b)** PCR analyses of *GLK* wild-type and mutant allele in the genomic DNA from mouse tails. The PCR products of the higher band (1400 bp) indicate wild-type (WT) allele, and the lower band (1000 bp) indicates *GLK* mutant allele. **(c–e)** Flow cytometry analyses of T lymphocytes from the thymus (c), spleen (d), and lymph nodes (e) of wild-type and *GLK*-deficient (*GLK*-KO) mice. Data are presented as mean ± s.e.m. **(f)** Immunoblot analyses of

GLK, p-PLC $\gamma$ 1, p-Lck, Lck and tubulin in lysates of mouse T cells stimulated with anti-CD3-biotin and streptavidin. **(g)** CFSE dilution assays of T cell proliferation in purified T cells. Proliferation indexes (mean  $\pm$  s.e.m) analyzed by FlowJo software are also shown. Data are representative of at least three independent experiments **(b-g)**.



**Supplementary Figure 7. Differentiation of  $T_H1$  and  $T_H2$  is impaired by GLK deficiency.** Flow cytometry of IFN- $\gamma$ -producing, IL-4-producing and Foxp3-positive  $CD4^+$  T cells. Data are presented as mean  $\pm$  s.e.m. Data are representative of at least three independent experiments.





**Supplementary Figure 8. Serum IL-17 levels are increased in SLE patients. (a)** Profile of SLE patients and paired healthy control. Data are presented as mean ± s.d. **(b)** ELISA assays of IL-17, TNF- $\alpha$ , and IL-6 in the sera of SLE patients and healthy controls (HC).