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The kinase GLK controls autoimmunity and NF-κB signaling by activating the kinase PKC-θ in T cells

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Protein kinase C-θ (PKC-θ) is required for activation of the transcription factor NF-κB induced by signaling via the T cell antigen receptor (TCR); however, the direct activator of PKC-θ is unknown. We report that the kinase GLK (MAP4K3) directly activated PKC-θ 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-θ 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-θ, and activation of GLK–PKC-θ–IKK could be used as new diagnostic biomarkers and therapeutic targets for systemic lupus erythematosus.

NF-κB 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-κB activation, which is involved in host defense against infection and the development of inflammation, cancer and autoimmunity. Protein kinase C-θ (PKC-θ) has a critical role in the activation of inhibitor of NF-κB (IκB) kinase (IKK) and NF-κB and functions of T cells[1]. The adaptor SLP-76 is required for PKC-θ activation after TCR stimulation[2,3]. However, the signal transduction from SLP-76 to PKC-θ and the direct kinase that activates PKC-θ have remained unclear. Thus, the most critical issue in TCR-induced NF-κB activation is the identification of the pivotal link between SLP-76 and PKC-θ.

PKC-θ activation requires phosphorylation at Thr538 (ref. 4). The kinase PDK1 interacts with PKC-θ, and phosphorylation of PKC-θ at Thr538 is defective in PDK1-deficient T cells[5]. Thus, PDK1 has been proposed to directly phosphorylate PKC-θ at Thr538, although no clear evidence exists that PDK1 directly phosphorylates PKC-θ *in vitro*. Furthermore, the observation that PDK1 can be activated only by the coreceptor CD28, and hence not by TCR signaling[5], further rules out the possibility that PDK1 is the direct kinase for PKC-θ activation induced by TCR signaling. Thus, the kinase that directly activates PKC-θ 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[6]. GLK contains a conserved N-terminal kinase domain, a conserved C-terminal citron homology domain and several proline-rich motifs in between[6]. Phosphorylation of the MAPK Jnk is induced by GLK via the MAPKs MEKK1 and MKK4 (SEK1) in response to stress stimulation[6]. GLK also regulates cell growth by activating the kinase mTOR downstream effectors S6K1 and 4E-BP1 in epithelial cell lines after amino acid treatment[7]. 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[8], 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-θ.

RESULTS

GLK directly phosphorylates and activates PKC-θ

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-κB 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-κB 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-θ, a critical regulator upstream of IKK–NF-κB and downstream of SLP-76 in TCR signaling. PKC-θ phosphorylation was regulated by GLK

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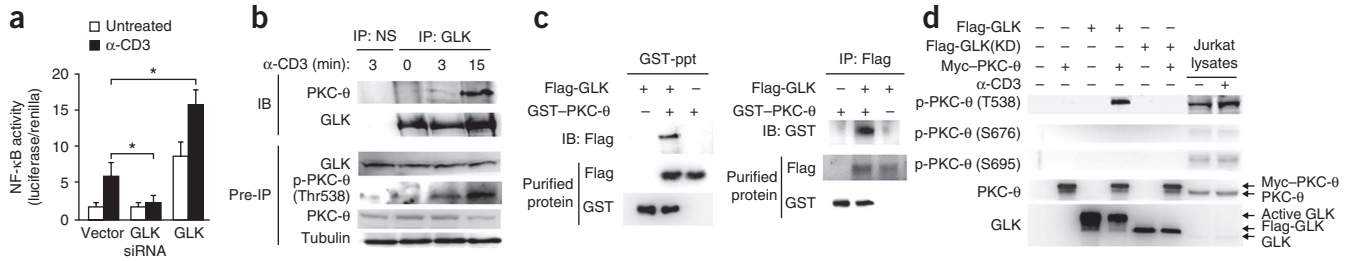

Figure 1 GLK directly interacts with and phosphorylates PKC-θ at Thr538. (**a**) Luciferase activity of an NF-κB reporter in Jurkat T cells transfected with vector alone, GLK-specific siRNA or plasmid encoding GLK. $*P < 0.01$ (two-tailed *t*-test). (**b**) Immunoprecipitation (IP) of endogenous GLK together with PKC-θ in lysates of mouse primary T cells after stimulation with anti-CD3 (α-CD3), analyzed by immunoblot (IB). NS, normal serum; p-, phosphorylated; Pre-IP, immunoblot analysis before immunoprecipitation. (**c**) *In vitro* binding assays of purified Flag-GLK and glutathione *S*-transferase (GST)–PKC-θ proteins. ppt, precipitation. Tubulin serves as a loading control throughout. (**d**) Immunoblot analysis of GLK expression and GLK-induced PKC-θ phosphorylation in *in vitro* kinase assays with Flag-GLK and Myc–PKC-θ (as the substrate) isolated from transfected HEK293T cells; far right, immunoblot analysis of PKC-θ in lysates of Jurkat cells (control). (**e**) Immunoblot analysis of GLK expression and GLK-induced PKC-θ phosphorylation in *in vitro* kinase assays with purified Flag-GLK and GST–PKC-θ. Data are representative of three independent experiments (error bars (**a**), s.d. of triplicate samples).

(**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[2]. 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

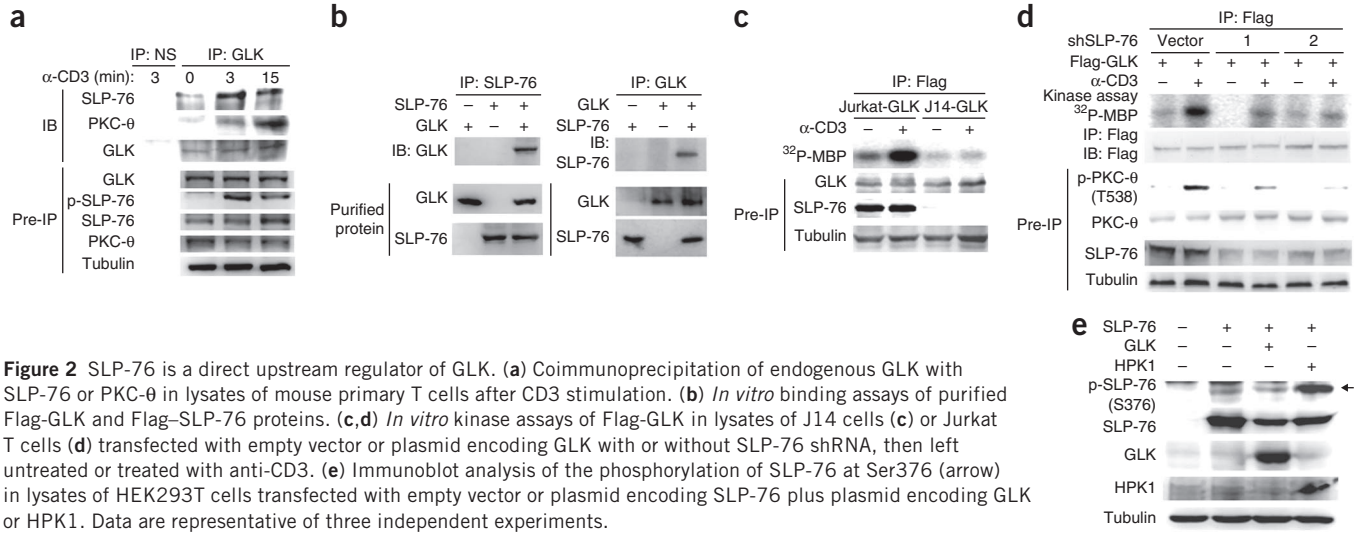

Figure 2 SLP-76 is a direct upstream regulator of GLK. (**a**) Coimmunoprecipitation of endogenous GLK with SLP-76 or PKC-θ in lysates of mouse primary T cells after CD3 stimulation. (**b**) *In vitro* binding assays of purified Flag-GLK and Flag–SLP-76 proteins. (**c**,**d**) *In vitro* kinase assays of Flag-GLK in lysates of J14 cells (**c**) or Jurkat T cells (**d**) transfected with empty vector or plasmid encoding GLK with or without SLP-76 shRNA, then left untreated or treated with anti-CD3. (**e**) Immunoblot analysis of the phosphorylation of SLP-76 at Ser376 (arrow) in lysates of HEK293T cells transfected with empty vector or plasmid encoding SLP-76 plus plasmid encoding GLK or HPK1. Data are representative of three independent experiments.

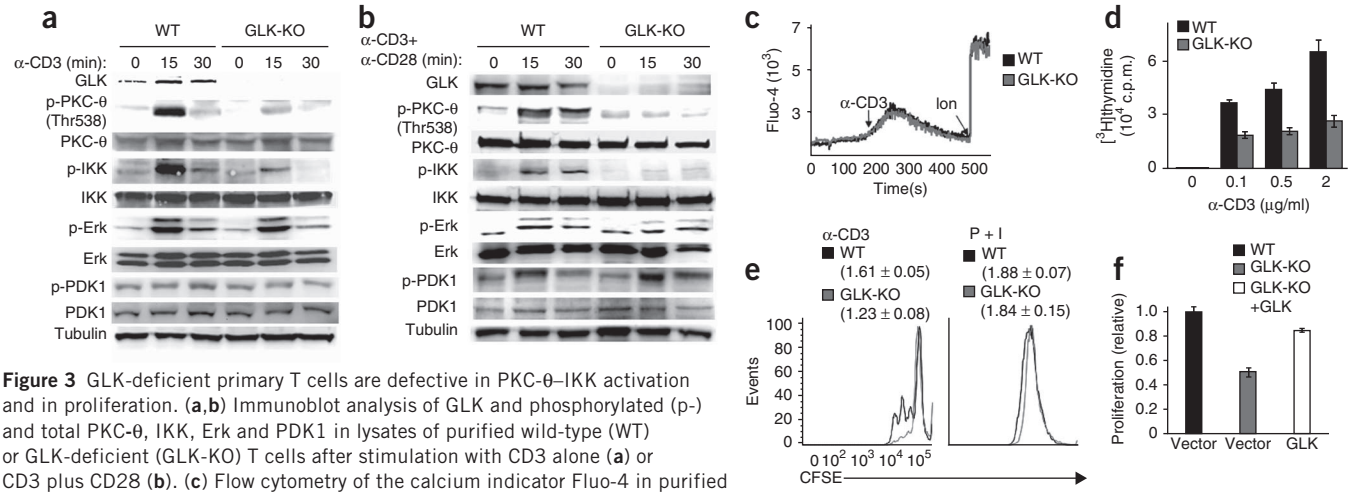

Figure 3 GLK-deficient primary T cells are defective in PKC-θ–IKK activation and in proliferation. (**a**,**b**) Immunoblot analysis of GLK and phosphorylated (p-) and total PKC-θ, IKK, Erk and PDK1 in lysates of purified wild-type (WT) or GLK-deficient (GLK-KO) T cells after stimulation with CD3 alone (**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-θ translocation[9], which suggests that Vav1 controls PKC-θ 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-θ and IKK was abolished in GLK-deficient T cells after stimulation with anti-CD3 (**Fig. 3a**) or with anti-CD3

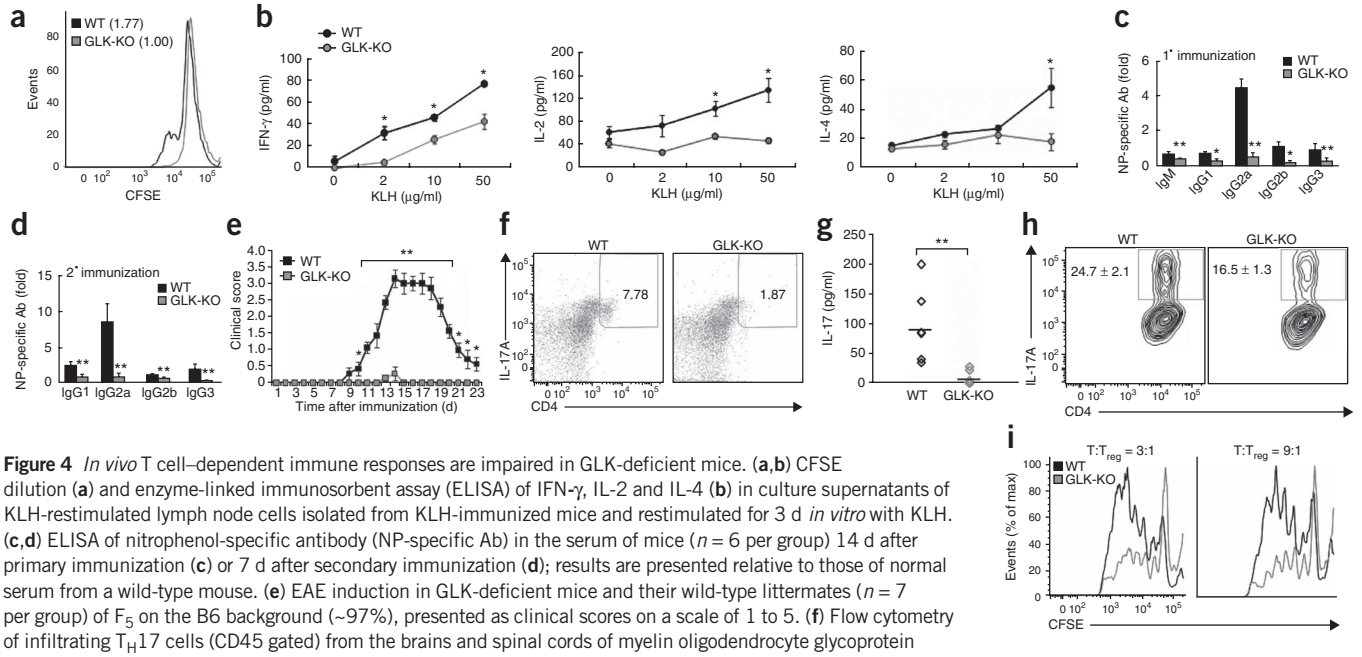

Figure 4 *In vivo* T cell–dependent immune responses are impaired in GLK-deficient mice. (**a**,**b**) CFSE dilution (**a**) and enzyme-linked immunosorbent assay (ELISA) of IFN-γ, IL-2 and IL-4 (**b**) in culture supernatants of KLH-restimulated lymph node cells isolated from KLH-immunized mice and 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_5 on the B6 background (~97%), presented as clinical scores on a scale of 1 to 5. (**f**) Flow cytometry of infiltrating T_H17 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 T_H17 cells. (**i**) Suppression of CFSE-labeled CD3$^+$ T cells by wild-type or GLK-deficient T_{reg} cells, presented as CFSE dilution in responding T cells cultured at a ratio of 3:1 (left) or 9:1 (right) with T_{reg} 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 ± s.e.m.).

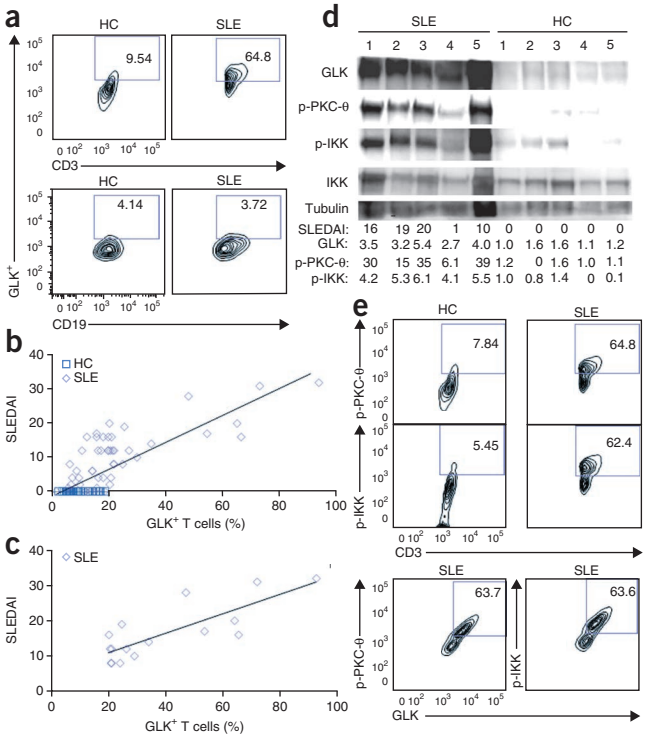

Figure 5 GLK expression and phosphorylation of PKC-θ at Thr538 are induced in T cells from people with SLE. (**a**) Flow cytometry analysis of GLK-expressing (GLK$^+$) lymphocytes from PBLs of people with SLE ($n = 49$) and unaffected controls (HC; $n = 35$); the results from one person with SLE (SLEDAI, 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$^+$ T cells from people with SLE with high frequency of GLK$^+$ cells (≥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-θ 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-θ 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-γ1, as well as Ca^{2+} signaling, were also unaffected (**Fig. 3c** and **Supplementary Fig. 6f**). As NF-κB signaling regulates T cell proliferation, we also studied the effect of GLK on T cell proliferation by analysis of [^{3}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-γ (IFN-γ), 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$^+$ lymphocytes of the interleukin 17 (IL-17)-producing helper T subset (T_H17 cells)[11]. PKC-θ deficiency results in ameliorated EAE and T_H17 responses[12,13]. Because GLK activates PKC-θ, we studied the role of GLK in T_H17-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 T_H17 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 T_H17 response in GLK-deficient mice was a T cell–intrinsic effect, we determined the *in vitro* T_H17-differentiation potential of GLK-deficient naive T cells. Consistent with the results obtained above, the *in vitro* differentiation of T_H17 cells, as well as of type 1 helper T cells (T_H1) and type 2 helper T cells (T_H2), was diminished by GLK deficiency (**Fig. 4h** and **Supplementary Fig. 7**). *In vitro* differentiation of regulatory T cells (T_{reg} cells) was not affected by GLK deficiency (**Supplementary Fig. 7**); however, GLK-deficient T_{reg} cells had greater suppressive activity *in vitro* (**Fig. 4i**). These results indicated that GLK deficiency *in vivo* protects mice from the development of T_H17-mediated autoimmune diseases. Defective TCR signaling and T_H17 differentiation, as well as enhanced T_{reg} cell function, of GLK-deficient T cells may have critical roles in the resistance of GLK-deficient mice.

GLK-induced PKC-θ activation in human autoimmune disease

T_H17-mediated inflammation also has an important role in the human autoimmune disease systemic lupus erythematosus (SLE)[14]. Because autoimmune induction and T_H17 responses were attenuated in GLK-deficient mice and because GLK activates the PKC-θ–IKK pathway, we studied whether the GLK–PKC-θ–IKK–IL-17 cascade is involved in human SLE. We examined GLK expression and PKC-θ–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 (≥21%

Table 1 Correlation of SLEDAI and GLK$^+$ T cell frequency in SLE

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

GLK$^+$ 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-θ–IKK activation. The frequency of T cells with phosphorylated PKC-θ 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-θ–IKK activation in people with SLE (**Fig. 5d**). These data suggested that GLK activates PKC-θ 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-θ in T cells.

DISCUSSION

A key finding of our study was the identification of GLK as a kinase that directly phosphorylated PKC-θ at Thr538 during TCR signaling. PKC-θ phosphorylation at residue Thr538 in the activation loop is essential to NF-κB activation in T cells[4]. GLK-induced phosphorylation of PKC-θ–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-θ 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_H1, T_H2 and T_H17 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-γ (a T_H1 cytokine) and IL-4 (a T_H2 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-θ-deficient T cells[13,15,16]. The resistance of GLK-deficient mice to EAE induction and diminished T_H17 responses are also consistent with the phenotype of PKC-θ-deficient mice[12,13]. The number of thymic natural T_{reg} cells was unaffected in GLK-deficient mice. However, T_{reg} cell numbers are impaired in mice deficient in PKC-θ, the scaffolding protein CARMA1 or the adaptor Bcl-10 or mice with mutant IKK2 because of impaired PKC-θ–NF-κB signaling[17–21]. PKC-θ can be activated by multiple signaling pathways such as CD28, CD4, CD8, integrin $\alpha_L\beta_2$ (LFA-1) or endoplasmic reticulum stress[22,23]. It remains unclear how PKC-θ–NF-κB signaling is activated during T_{reg} cell development. Our data indicates that GLK activates PKC-θ by phosphorylating the Thr538 residue but not other known phosphorylation sites (such as Ser676 or Ser695). GLK is probably not the only PKC-θ-activating kinase. Therefore, it is likely that PKC-θ–CARMA1–Bcl-10–IKK2 can still be activated by other kinases in T_{reg} cells, leading to normal numbers of T_{reg} cells in GLK-deficient mice. Although T_{reg} cell numbers were unaffected in GLK-deficient mice, T_{reg} cell activity was enhanced by GLK deficiency. PKC-θ has a negative role in T_{reg} cell–mediated suppression[24]. The role of GLK in regulating T_H1, T_H2, T_H17 and T_{reg} cells was essentially consistent with that of PKC-θ, which supports the idea that GLK is probably the sole activator of PKC-θ 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-θ–IKK activation, which suggested that activation of the GLK–PKC-θ–IKK pathway controls SLE pathogenesis. This is the first report to our knowledge of GLK overexpression and PKC-θ–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[14]. 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-θ–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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1. McCully, R.R. & Pomerantz, J.L. The protein kinase C-responsive inhibitory domain of CARD11 functions in NF-κB activation to regulate the association of multiple signaling cofactors that differentially depend on Bcl10 and MALT1 for association. *Mol. Cell. Biol.* **28**, 5668–5686 (2008).
2. Herndon, T.M., Shan, X.C., Tsokos, G.C. & Wange, R.L. ZAP-70 and SLP-76 regulate protein kinase C-θ and NF-κB activation in response to engagement of CD3 and CD28. *J. Immunol.* **166**, 5654–5664 (2001).
3. Koretzky, G.A., Abtahian, F. & Silverman, M.A. SLP76 and SLP65: complex regulation of signalling in lymphocytes and beyond. *Nat. Rev. Immunol.* **6**, 67–78 (2006).

4. Liu, Y., Graham, C., Li, A., Fisher, R.J. & Shaw, S. Phosphorylation of the protein kinase C-θ activation loop and hydrophobic motif regulates its kinase activity, but only activation loop phosphorylation is critical to *in vivo* nuclear-factor-κB induction. *Biochem. J.* **361**, 255–265 (2002).
5. Park, S.G. *et al.* The kinase PDK1 integrates T cell antigen receptor and CD28 coreceptor signaling to induce NF-κB and activate T cells. *Nat. Immunol.* **10**, 158–166 (2009).
6. Diener, K. *et al.* Activation of the c-Jun N-terminal kinase pathway by a novel protein kinase related to human germinal center kinase. *Proc. Natl. Acad. Sci. USA* **94**, 9687–9692 (1997).
7. Findlay, G.M., Yan, L., Procter, J., Mieulet, V. & Lamb, R.F.A. MAP4 kinase related to Ste20 is a nutrient-sensitive regulator of mTOR signalling. *Biochem. J.* **403**, 13–20 (2007).
8. Shui, J.W. *et al.* Hematopoietic progenitor kinase 1 negatively regulates T cell receptor signaling and T cell-mediated immune responses. *Nat. Immunol.* **8**, 84–91 (2007).
9. Dienz, O. *et al.* Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa and phospholipase Cγ1 are required for NF-κB activation and lipid raft recruitment of protein kinase C-θ induced by T cell costimulation. *J. Immunol.* **170**, 365–372 (2003).
10. Di Bartolo, V. *et al.* A novel pathway down-modulating T cell activation involves HPK-1-dependent recruitment of 14–3-3 proteins on SLP-76. *J. Exp. Med.* **204**, 681–691 (2007).
11. Lock, C. *et al.* Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. *Nat. Med.* **8**, 500–508 (2002).
12. Salek-Ardakani, S., So, T., Halteman, B.S., Altman, A. & Croft, M. Protein kinase C θ controls Th1 cells in experimental autoimmune encephalomyelitis. *J. Immunol.* **175**, 7635–7641 (2005).
13. Tan, S.L. *et al.* Resistance to experimental autoimmune encephalomyelitis and impaired IL-17 production in protein kinase C θ–deficient mice. *J. Immunol.* **176**, 2872–2879 (2006).
14. Yang, J. *et al.* Th17 and natural T_{reg} cell population dynamics in systemic lupus erythematosus. *Arthritis Rheum.* **60**, 1472–1483 (2009).
15. Anderson, K. *et al.* Mice deficient in PKCθ demonstrate impaired *in vivo* T cell activation and protection from T cell–mediated inflammatory diseases. *Autoimmunity* **39**, 469–478 (2006).
16. Healy, A.M. *et al.* PKC-θ–deficient mice are protected from Th1-dependent antigen-induced arthritis. *J. Immunol.* **177**, 1886–1893 (2006).
17. Medoff, B.D. *et al.* Differential requirement for CARMA1 in agonist-selected T-cell development. *Eur. J. Immunol.* **39**, 78–84 (2009).
18. Barnes, M.J. *et al.* Commitment to the regulatory T cell lineage requires CARMA1 in the thymus but not in the periphery. *PLoS Biol.* **7**, e51 (2009).
19. Schmidt-Supprian, M. *et al.* Mature T cells depend on signaling through the IKK complex. *Immunity* **19**, 377–389 (2003).
20. Gupta, S. *et al.* Differential requirement of PKC-θ in the development and function of natural regulatory T cells. *Mol. Immunol.* **46**, 213–224 (2008).
21. Schmidt-Supprian, M. *et al.* Differential dependence of $CD4^+CD25^+$ regulatory and natural killer–like T cells on signals leading to NF-κB activation. *Proc. Natl. Acad. Sci. USA* **101**, 4566–4571 (2004).
22. Sakaki, K. & Kaufman, R.J. Regulation of ER stress-induced macroautophagy by protein kinase C. *Autophagy* **4**, 841–843 (2008).
23. Hayashi, K. & Altman, A. Protein kinase C θ (PKC θ): a key player in T cell life and death. *Pharmacol. Res.* **55**, 537–544 (2007).
24. Zanin-Zhorov, A. *et al.* Protein kinase C-θ mediates negative feedback on regulatory T cell function. *Science* **328**, 372–376 (2010).

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 CD3ε (145-2C11) and anti-human CD3ε (OKT3) were purified from mouse ascites by protein A–Sepharose chromatography. Anti-GLK[6], 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-θ phosphorylated at Thr538 (07-885) and to PKC-θ phosphorylated at Ser695 (07-885) were from Upstate Biotechnology. Anti-PKC-θ (C-19), antibody to PKC-θ phosphorylated at Ser676 (sc-33024), ant-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-θ 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[6,8]. Myc-tagged PKC-θ, 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-κB reporter plasmid (pNF-κB-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-θ 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 μg/ml of anti-CD3 for the appropriate time at 37 °C. For induction of primary T cell activation, 3×10^6 purified T cells were stimulated with 3 μg/ml biotin-conjugated anti–CD3 (500A2; eBioscience) plus 3 μg/ml of streptavidin (Sigma).

***In vitro* kinase assays.** Flag-GLK was immunoprecipitated from unstimulated or anti-CD3-stimulated Jurkat or J14T cell lysates (80 μg) 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 μM nonradioactive adenosine triphosphate (ATP), 4 μg myelin basic protein and with or without 10 μCi of [γ-^{32}P]ATP in 35 μl 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 μl 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-γ (XMG1.2), phycoerythrin–anti-IL-4 (11B11) and phycoerythrin–anti-IL-17A (TC11-18H10.1). Pacific blue–anti-MCD4 (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_H1 and T_H2 cytokines from immunized mice was measured as described[8]. EAE was induced as described[8].

***In vitro* T cell–differentiation assays.** $CD4^+CD25^-$ cells were purified from the lymph nodes of mice. Cells (2.5×10^5) were cultured in 500 μl medium in 48-well plates coated with anti-CD3 (2 μg/ml) and anti-CD28 (3 μg/ml). For T_{reg} cell differentiation, cells were cultured in medium containing 10 ng/ml of IL-2, 10 ng/ml of TGF-β (transforming growth factor beta), 2.5 μg/ml of anti–IL-4 and 2.5 μg/ml of anti–IFN-γ. For T_H17 differentiation, cells were cultured in medium containing 20 ng/ml of IL-6, 5 ng/ml of TGF-β, 50 ng/ml of interleukin 23 (IL-23), 5 μg/ml of anti–IL-4 and 5 μg/ml of anti–IFN-γ. For T_H1 differentiation, cells were cultured in medium containing 5 ng/ml of IL-12 and 1 μg/ml of anti–IL-4. For T_H2 differentiation, cells were cultured in medium containing 10 ng/ml of IL-4 and 1 μg/ml of anti–IFN-γ.

***In vitro* suppression assays.** Mouse $CD4^+$ T cells were negatively selected from the spleen and lymph nodes of mice. In the second round of purification, $CD4^+CD25^+$ T_{reg} cells were isolated from the $CD4^+$ T cells using magnetically coupled antibodies to mouse CD25. T_{reg} cells were added to $CD3^+$ T cells (at a final concentration of 2×10^5 cells per 500 μl) 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.