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Induction of pathogenic Th17 cells by inducible salt sensing kinase SGK1

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Abstract

Th17 cells are highly proinflammatory cells critical for clearing extracellular pathogens and for induction of multiple autoimmune diseases¹. IL-23 plays a critical role in stabilizing and reinforcing the Th17 phenotype by increasing expression of IL-23 receptor (IL-23R) and endowing Th17 cells with pathogenic effector functions^{2, 3}. However, the precise molecular mechanism by which IL-23 sustains the Th17 response and induces pathogenic effector functions has not been elucidated. Here, we used transcriptional profiling of developing Th17 cells to construct a model of their signaling network and nominate major nodes that regulate Th17 development. We identified serum glucocorticoid kinase-1 (SGK1), a serine-threonine kinase⁴, as an essential node downstream of IL-23 signaling. SGK1 is critical for regulating IL-23R expression and stabilizing the Th17 cell phenotype by deactivation of Foxo1, a direct repressor of IL-23R expression. SGK1 has been shown to govern Na⁺ transport and salt (NaCl) homeostasis in other cells^{5, 6, 7, 8}. We here show that a modest increase in salt concentration induces SGK1 expression, promotes IL-23R expression and enhances Th17 cell differentiation *in vitro* and *in vivo*, accelerating the development of autoimmunity. Loss of SGK1 abrogated Na⁺-mediated Th17 differentiation in an IL-23-dependent manner. These data demonstrate that SGK1 plays a critical role in the induction of pathogenic Th17 cells and provides a molecular insight into a mechanism by which an environmental factor such as a high salt diet triggers Th17 development and promotes tissue inflammation.

To determine the molecular mechanisms by which naïve T cells develop into effector Th17 cells, we measured genome-wide mRNA expression profiles using microarrays along 18 time points over 72 hours following the *in vitro* exposure of naive T cells to Th17 polarizing conditions (TGF-β1 with IL-6). To examine the role of IL-23 in Th17 development, we added IL-23 at the late time points (48 – 72 h) and monitored the transcriptional response in both wild-type (WT) and *Il23r*^{-/-} cells. We ranked the genes according to their extent of induction in cells treated with TGF-β1 and IL-6 (relative to nonpolarized activated T cells)

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Authors' contribution

C.W, N.Y. and T.T. performed experiments and wrote the manuscript. C.Z., S.X. and Y.K. performed experiments. N.Y. analyzed the data. A.R. and V.K.K. supervised the study and edited the manuscript.

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and repression in *Ii23r^{-/-}* cells (relative to WT cells) (**Methods**; Fig. 1a and Supplementary Table 1). SGK1 was one of the top ranking genes, whose transcriptional regulation is strongly associated with both IL-23R signaling and Th17 cell differentiation (Fig. 1a). qPCR analysis showed that SGK1 is induced at low levels by TGF- β 1 (iTreg), and not induced in other T cell subsets (Th0, Th1, Th2). As expected, it is most highly expressed under Th17 differentiation conditions (Fig. 1b). SGK1 expression is strongly induced during the first two hours following stimulation of naïve T cells under Th17-polarizing conditions. This is followed by a sharp decline by 10 hours to a steady expression level that is still substantially higher than in the control population (Fig. 1c and Supplementary Fig. 1a). Furthermore, SGK1 expression is specifically induced and maintained by exposure to IL-23 (Fig. 1c and Supplementary Fig. 1b). While *Ii23r^{-/-}* T cells initially produce SGK1 mRNA, they cannot sustain this expression (Supplementary Fig. 1b, c). Finally, the kinase activity of SGK1 is also significantly higher in Th17 cells than in other T cell subsets (Supplementary Fig. 1d), and restimulation of differentiated Th17 cells with IL-23 further elevates SGK1 kinase activity (Supplementary Fig. 1e). Thus, IL-23 signaling is critical for maintaining SGK1 expression during Th17 cell differentiation.

Network analysis⁹ of the transcriptional changes in *Ii23r^{-/-}* T cells singled out SGK1 as a potential nodal point downstream of IL-23R signaling. Based on a curated database of protein-protein interactions (PPI), we constructed a network model that connects known proteins of the IL-23R signaling pathway (**Methods**) to the transcription factors whose function is dysregulated in *Ii23r^{-/-}* cells (**Methods**; Fig. 1d and Supplementary Fig. 1f). We ranked the network's nodes based on a centrality measure, defined as the fraction of IL-23R-affected transcription factors downstream of that node in the network (**Methods**; Supplementary Table 1). SGK1 was the highest-ranking node (Supplementary Fig. 1g), suggesting that it acts both as a transcriptional target of IL-23R signaling and as a kinase that may mediate the transcriptional effects of the pathway.

Using *Sgk1^{-/-}* mice, we studied the impact of loss of SGK1 on Th17 differentiation *in vitro*. We observed no abnormality of SGK1-deficient T cells during primary differentiation into Th17 cells (Fig. 1e). However, *Sgk1^{-/-}* Th17 cells restimulated with IL-23 showed impaired IL-17 production (Fig. 1e and Supplementary Fig. 2b). Memory *Sgk1^{-/-}* T cells also showed a defect in IL-17 production upon IL-23 stimulation, but not under stimulation with TGF- β 1 and IL-6 (Supplementary Fig. 2a). To study the function of SGK1 specifically in IL-17-producing CD4⁺ T cells, we generated *Ii17^{Cre}Sgk1^{fl/fl}* mice. *Ii17^{Cre}Sgk1^{fl/fl}* T cells also showed no defect in primary Th17 differentiation, but displayed reduced IL-17 production upon restimulation with IL-23 (Fig. 1f and Supplementary Fig. 2c). IL-23R expression was also significantly reduced in *Sgk1^{-/-}* T cells (Fig. 1g). Thus, loss of SGK1 does not affect primary Th17 differentiation, but profoundly affects their stability and IL-23R expression. One possible explanation for the dispensability of SGK1 during primary Th17 differentiation is redundancy with other kinases, such as its homolog AKT¹⁰. However, SGK1 seems to be indispensable for IL-23R-dependent stability and maintenance of Th17 cells.

Microarray analysis of *Sgk1^{-/-}* vs. WT Th17 cells restimulated with IL-23 showed a significant overlap in differentially expressed genes with the *Ii23r^{-/-}* vs. WT IL-23-restimulated Th17 cell profiles, further supporting the functional relatedness of the SGK1 and IL-23R pathways (Fisher exact test, $p < 10^{-3}$) (Fig. 1h and Supplementary Fig. 2d). Consistently, genes downregulated in *Sgk1^{-/-}* cells are significantly enriched (Fisher exact test, $p < 10^{-6}$) for genes that are upregulated in WT Th17 cells compared to other T cell subsets¹¹ (**Methods** and Supplementary Fig. 2e). Selected genes were confirmed by qPCR analysis (Supplementary Fig. 2f). Genes from several other pathways are also enriched (over- or underexpressed) (Supplementary Table 2), including cell cycle and proliferation,

which may be related to the known role of SGK1 as a regulator of proliferation and apoptosis^{7, 8, 10}. Although our analysis strongly associates SGK1 with the Th17 program, genes important for development and function of other T cell subsets, such as *Ifng*, *Tbx21* or *Gata3* were also dysregulated in *Sgk1*^{-/-} cells, suggesting possible additional effects of this kinase in other T cell subsets.

To determine the role of SGK1 *in vivo*, we immunized *Cd4*^{Cre}*Sgk1*^{fl/fl} mice with MOG₃₅₋₅₅ to induce experimental autoimmune encephalomyelitis (EAE). SGK1-deficient mice exhibited significantly reduced EAE incidence and severity. IL-17 production from infiltrated CD4⁺ T cells in different organs of SGK1-deficient mice was also reduced, whereas IFN- γ levels were unaffected (Fig. 2a and Supplementary Fig. 3a). When we restimulated the isolated T cells from immunized mice with IL-23 in the presence of MOG₃₅₋₅₅, the SGK1-deficient T cells also exhibited impaired IL-17 but normal IFN- γ production (Supplementary Fig. 3b, c). Next, using *I123*^{gfp} reporter mice, we observed reduced IL-23R (GFP) expression on infiltrating CD4⁺ T cells in different organs of SGK1-deficient mice undergoing EAE (Supplementary Fig. 4a). Consistent with *Cd4*^{Cre}*Sgk1*^{fl/fl} mice, reduced Th17 differentiation and disease severity were also observed in *I117*^{Cre}*Sgk1*^{fl/fl} mice during EAE (Fig. 2b). In addition, to exclude any effects of SGK1-deficient bystander cells, we transferred purified *I117*^{Cre}*Sgk1*^{fl/fl} CD4⁺ T cells into *Rag2*^{-/-} mice and induced EAE. Mice that received SGK1-deficient T cells developed attenuated disease as compared to mice that received WT T cells (Supplementary Fig. 4b).

To determine why we observed fewer Th17 cells in SGK1-deficient mice, we transferred purified GFP⁺ cells from differentiated *Cd4*^{Cre}*Sgk1*^{fl/fl}*I117a*^{gfp} or control Th17 cells to congenic Ly5.1 mice and traced the IL-17 GFP⁺ cells in different organs following immunization with MOG₃₅₋₅₅ (Fig. 2c). Starting with the same number of CD4⁺IL-17⁺ T cells, we found that 7 and 12 days after transfer, SGK1-deficient Th17 cells failed to maintain IL-17 production, especially in the central nervous system (CNS) (Fig. 2d and Supplementary Fig. 4c). Next, we crossed *I117*^{Cre}*R26*^{YFP} mice onto the SGK1-deficient background, and analyzed the expression of IL-17 in T cells that had turned on the *I117f* gene as determined by eYFP-expression. We induced EAE in these mice and analyzed the frequency of eYFP⁺ cells producing IL-17 in infiltrating CD4⁺ T cells in the lymph nodes (LN) and CNS. The *Sgk1*^{-/-} reporter mice exhibited a smaller proportion of CD4⁺eYFP⁺ T cells in both organs. Furthermore, there was a dramatic loss of IL-17 expression by eYFP⁺ T cells in the SGK1-deficient mice, indicating that Th17 cells could not stably retain IL-17 production during EAE (Fig. 2e and Supplementary Fig. 4d).

To better understand the molecular role of SGK1 in Th17 cells, we conducted another network analysis, using PPI data to connect SGK1 to the transcription factors whose activity is dysregulated in *Sgk1*^{-/-} Th17 cells (**Methods**). The analysis suggested Foxo1 as one of the highest-ranking nodes downstream of SGK1 (Fig. 3a; Supplementary Table 1 and Supplementary Fig. 5a). Foxo1 phosphorylation by SGK1 has previously been shown in adipocytes to lead to its deactivation and translocation from the nucleus to the cytoplasm¹². Consistent with this observation, we have observed that SGK1 phosphorylates Foxo1 (Supplementary Fig. 5b). By immunoblot of *Sgk1*^{-/-} Th17 cells restimulated with IL-23, we confirmed that not only is there reduced phosphorylation of Foxo1 in the nucleus, but there was increased mRNA and protein expression of Foxo1 (Fig. 3b, c), suggesting that compromised phosphorylation of Foxo1 can result in its own transcriptional upregulation. It has been previously shown that Foxo1 can regulate its own expression¹³ and we have found that Foxo1 binds to a site located about 1 kilobase (kb) upstream of the first exon in the *Foxo1* locus (Supplementary Fig. 6a). Transfection of a *Foxo1* luciferase reporter in the presence of Foxo1 led to increased luciferase activity (Supplementary Fig. 6b), while increasing expression of SGK1 in the presence of Foxo1 resulted in a dose-dependent

decrease in reporter activity, suggesting that SGK1 inhibited Foxo1-mediated transactivation of its own promoter (Fig. 3d).

To decipher the consequences of Foxo1 expression on Th17 cell development, we used *Foxo1*^{-/-} CD4⁺ memory T cells and observed increased expression of IL-23R and IL-17A compared to WT cells, indicating that Foxo1 may act as a repressor of Th17 cell development and of IL-23 signaling (Fig. 3e and Supplementary Fig. 6c). We also found potential binding sites of Foxo1 located about 1 kb upstream of the first exon of the *Il23r* locus by ChIP-PCR (Supplementary Fig. 6d). Moreover, there is significantly enriched binding of Foxo1 on the *Il23r* promoter region in *Sgk1*^{-/-} cells compared to WT T cells, indicating enhanced suppression of *Il23r* transcription in the absence of SGK1 (Fig. 3f). ROR γ t has been suggested to be the master transcription factor of Th17 development and ChIP-seq²⁸ and our ChIP-PCR analysis confirmed that IL-23R is one of the targets of ROR γ t (Supplementary Fig. 6e). Indeed, we observed that the *Il23r* promoter is transactivated by ROR γ t in IL-23-restimulated Th17 cells and it can be inhibited by Foxo1 in a dose-dependent manner (Supplementary Fig. 6f, g). While Foxo1 inhibited ROR γ t-mediated *Il23r* expression, co-expression of SGK1 together with ROR γ t and Foxo1 abrogated the suppressive effects of Foxo1 and rescued *Il23r* promoter transcriptional activity (Fig. 3g). Additionally, the inhibition of *Il23r* transcription by a phosphorylation-insensitive triple alanine mutant of Foxo1, Foxo1 AAA, was not reduced in the presence of SGK1 (Supplementary Fig. 6h). Furthermore, we observed an endogenous Foxo1-ROR γ t interaction in primary Th17 cells (Fig. 3h). These data support a model whereby some of the effects of SGK1 are due to phosphorylation of Foxo1, which may be a key step in relieving ROR γ t from Foxo1-mediated inhibition, enhancing the expression of IL-23R.

SGK1 has been reported to act as a mediator for sodium homeostasis. It can be induced by exogenous sodium chloride and is one of the major kinases that regulates Na⁺ intake by phosphorylation of epithelial sodium channels (ENaCs)^{4, 5}. Considering the defects in Th17 development in *Sgk1*^{-/-} mice, this raised the hypothesis that increasing sodium concentration may affect the Th17 cell phenotype through SGK1. To test this hypothesis, we first activated naive T cells in the presence of additional NaCl, but in the absence of any polarizing cytokines. Microarray analysis of these NaCl-treated cells showed a significant upregulation of *Sgk1* and of multiple other genes associated with Th17 development (Fisher exact test; $p < 10^{-3}$; Supplementary Table 2; Supplementary Fig. 7a), which we confirmed by qPCR analysis of selected genes (Supplementary Fig. 7b). We also observed increased mRNA and protein levels of IL-17 and IL-23R with additional NaCl under various Th17 polarizing conditions (Fig. 4a, b and Supplementary Fig. 7c). Furthermore, a sodium-induced increase in Th17 development and IL-23R expression was not observed in SGK1-deficient T cells, specifically in the context of IL-23-IL-23R signaling (Fig 4c, d). Importantly, culturing cells with mannitol did not alter Th17 cell differentiation, excluding the possibility that the Th17 program is initiated simply by the alteration of osmotic pressure (Supplementary Fig. 7d).

Recent studies have shown that different components in the daily diet and gut microbiota can strongly affect the frequency of effector T cells in the gut^{14, 15}. Additionally, previous data indicate that molecules related to sodium homeostasis can influence Th17 cell responses^{16, 17}. To further understand the effect of NaCl on Th17 cell generation *in vivo*, we fed a high salt diet (HSD) to WT or *Cd4*^{Cre}*Sgk1*^{fl/fl} mice. After 3 weeks on HSD, we observed that unimmunized WT mice showed a marked increase in the frequency of Th17 cells in the lamina propria (LP), while no notable changes were observed in the mesenteric lymph nodes (mLN) or spleen. On the other hand, SGK1-deficient mice exhibited a much milder enhancement of Th17 cell frequency in the gut while there was no increase in IFN- γ production in any of the mice fed with HSD (Supplementary Fig. 8a, b).

Finally, we studied whether HSD would affect the development of Th17 and EAE *in vivo*. Mice fed HSD showed increased EAE severity when compared to the WT mice, which was dramatically reduced in SGK1-deficient mice (Fig. 4e and Supplementary Fig. 8c,d). We also observed a significantly higher frequency of Th17 cells in mLN and CNS of WT mice fed with HSD, but not in SGK1-deficient mice. The percentage of IFN- γ producing T cells in the CNS, but not in the peripheral immune compartments, of WT mice was increased in mice fed HSD, suggesting that HSD may increase infiltration, but not expansion of IFN- γ ⁺ effector T cells, in the target organs (Fig. 4f and Supplementary Fig. 8e). Consistent with our *in vitro* data, we observed elevated IL-17 but not IFN- γ , production from CD4⁺ T cells isolated from EAE immunized WT mice fed with HSD and restimulated *in vitro* with MOG₃₅₋₅₅ (Supplementary Fig. 8f). The data presented here indicates that high sodium intake potentiates Th17 cell generation *in vivo* in an SGK1-dependent manner and therefore has the potential to increase the risk of promoting autoimmunity.

In conclusion, we used a combination of microarray data analysis, large-scale protein-protein interaction network analysis and experimental data from multiple different knockout mice to establish IL-23R-SGK1-Foxo1 as a critical axis in Th17 stabilization. We show that Foxo1 acts as a repressor of IL-23R expression by directly binding to the *Il23r* promoter and inhibiting ROR γ t mediated *Il23r* transactivation. Phosphorylation of Foxo1, mediated by SGK1, leads to its deactivation and promotes unopposed ROR γ t-mediated *Il23r* transcription. SGK1 has been extensively studied in the context of NaCl transport^{18, 19}. Modest increase of the NaCl concentration induces SGK1 expression in T cells with increased IL-23R expression and Th17 cell generation *in vitro*. Interestingly, even in unimmunized mice, enhancement of Th17 differentiation was observed *in vivo* in the gut and gut-associated lymphoid tissue and this increase in Th17 cells can be recalled at other peripheral sites following immunization. Although our data suggests an essential role for SGK1 in this process, it is likely that other immune cells and pathways are also influenced by increased salt intake. Furthermore, our result does not exclude additional alternative mechanisms by which an increase in NaCl affects Th17 cells. Nevertheless, the elevated *in vivo* Th17 differentiation by HSD raises the important issue of whether increased salt in the western diet and in processed foods is responsible for an increased generation of pathogenic Th17 cells and for an unprecedented increase in autoimmune diseases in the western world.

Methods Summary

Microarrays and network analysis

For gene expression analysis Affymetrix microarray chips were used. Data was processed using the GenePattern suite²⁰. Differentially expressed genes were detected using fold-change and t-test analysis (for *Sgk1*^{-/-} and NaCl-treated T cells), a consensus of fold-change, the EDGE software²¹ and a novel sigmoid-based method²² (for the *Il23r*^{-/-} Th17 cell time course data). A command-line version of the ANAT software⁹ was used for network analysis.

In vitro T cell differentiation

Naïve T cells were FACS-sorted, stimulated with plate bound anti-CD3/CD28 and the indicated cytokines or NaCl and cells were analyzed by qPCR or flow cytometry at different time points.

EAE model

Mice were immunized subcutaneously with MOG₃₅₋₅₅, CFA, heat-inactivated *Mycobacterium tuberculosis* and with intraperitoneal injection of *Bordetella pertussis* toxin.

***In vivo* cell transfer**

Naïve T cells were differentiated towards Th17 cells, then transferred into MOG_{35–55}/CFA immunized hosts and T cells isolated from various organs were analyzed by flow cytometry at 7–12 days after onset of EAE.

Western Blot/Immunoprecipitation

Differentiated T cells or transfected HEK293T cells were lysed in WCE buffer and lysates were subjected to Western blot or immunoprecipitation analysis.

Promoter activity reporter assay

HEK 293T cells were transfected with luciferase reporter constructs and expression vectors and luciferase expression was determined after 48 h.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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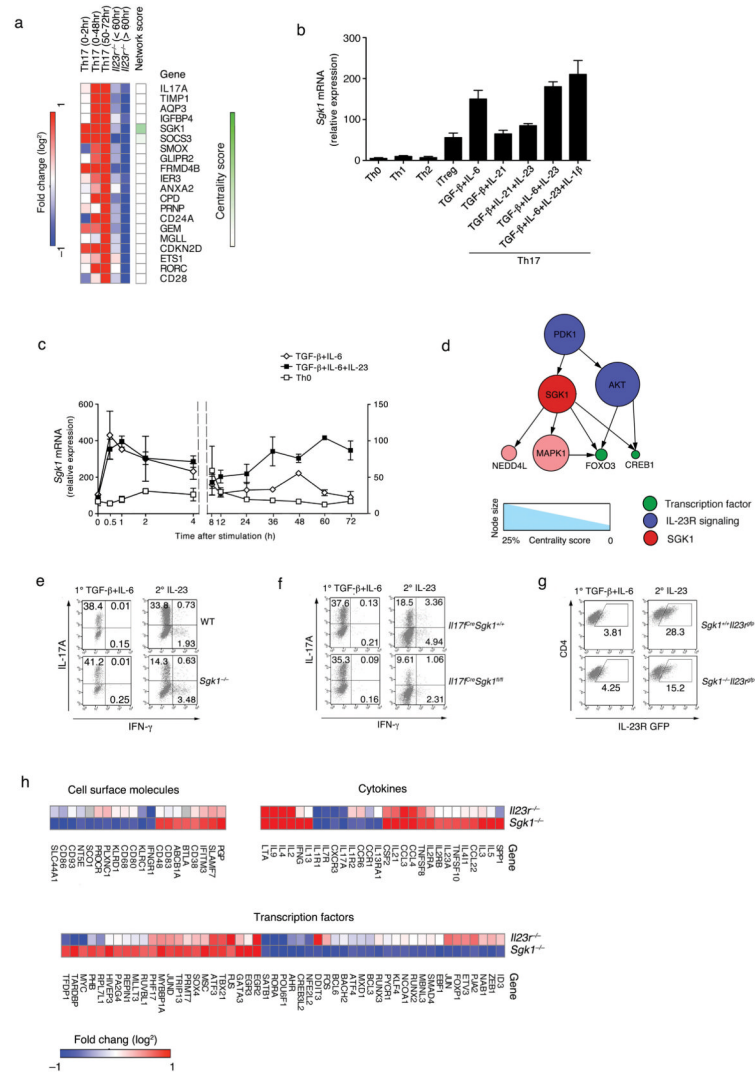


Figure 1.

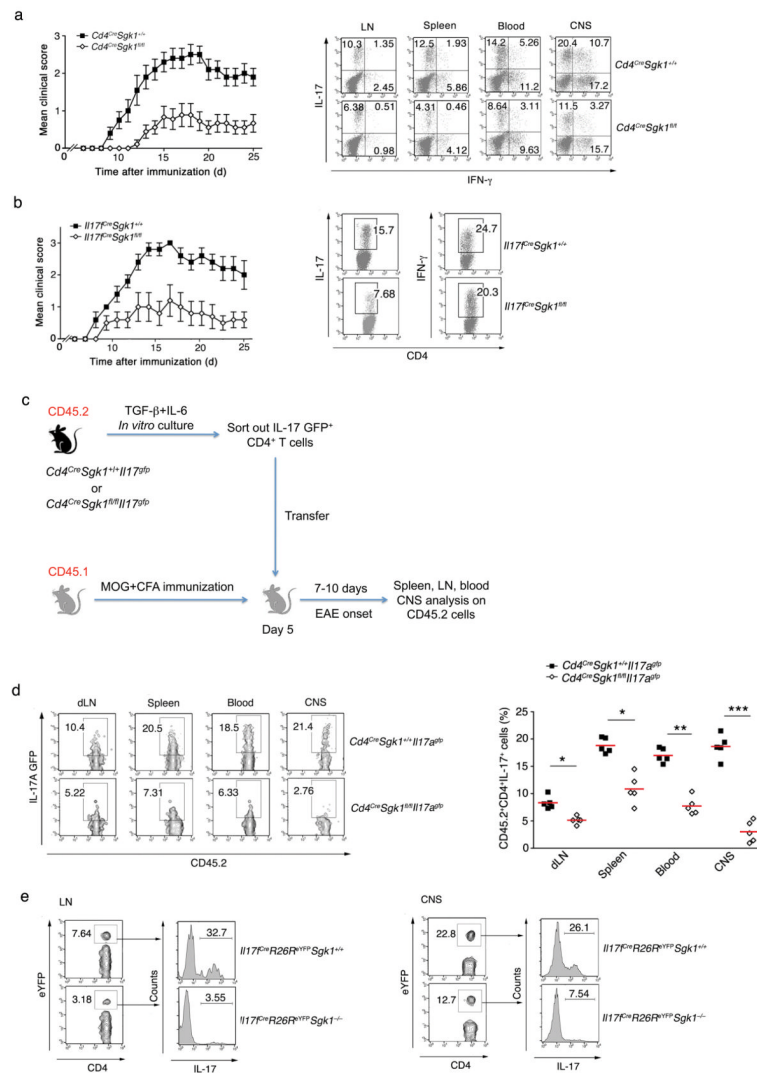


Figure 2.

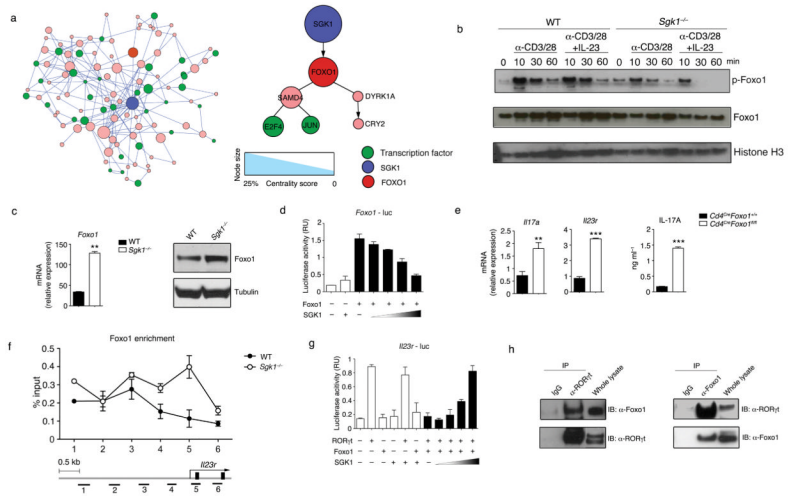


Figure 3.

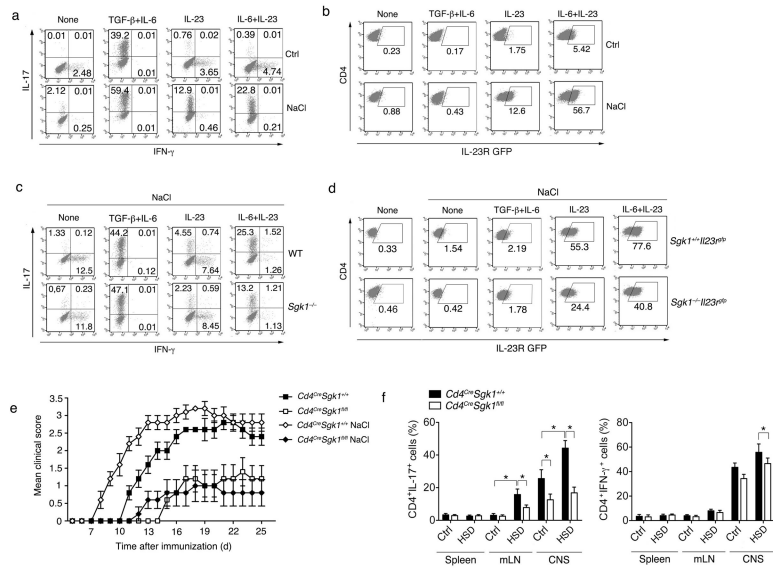


Figure 4.