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ARTICLE

Protein kinase 2 (CK2) controls CD4$^+$ T cell effector function in the pathogenesis of colitis

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Crohn's disease (CD), one of the major forms of inflammatory bowel disease (IBD), is characterized by chronic inflammation of the gastrointestinal tract and associated with aberrant CD4$^+$ T-helper type 1 (Th1) and Th17 responses. Protein kinase 2 (CK2) is a conserved serine–threonine kinase involved in signal transduction pathways, which regulate immune responses. CK2 promotes Th17 cell differentiation and suppresses the generation of Foxp3$^+$ regulatory T cells. The function of CK2 in CD4$^+$ T cells during the pathogenesis of CD is unknown. We utilized the T cell-induced colitis model, transferring CD45RB^{hi}-naive CD4$^+$ T cells from CK2α$^{fl/fl}$ controls and CK2α$^{fl/fl}$dLck-Cre mice into Rag1$^{-/-}$ mice. CD4$^+$ T cells from CK2α$^{fl/fl}$dLck-Cre mice failed to induce wasting disease and significant intestinal inflammation, which was associated with decreased interleukin-17A-positive (IL-17A$^+$), interferon-γ-positive (IFN-γ$^+$), and double-positive IL-17A$^+$IFN-γ$^+$ CD4$^+$ T cells in the spleen and colon. We determined that CK2α regulates CD4$^+$ T cell proliferation through a cell-intrinsic manner. CK2α is also important in controlling CD4$^+$ T cell responses by regulating NFAT2, which is vital for T cell activation and proliferation. Our findings indicate that CK2α contributes to the pathogenesis of colitis by promoting CD4$^+$ T cell proliferation and Th1 and Th17 responses, and that targeting CK2 may be a novel therapeutic treatment for patients with CD.

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INTRODUCTION

Protein kinase 2 (CK2) is a highly conserved serine–threonine kinase that is expressed in all eukaryotic organisms.[1] CK2 is responsible for the phosphorylation of serine and threonine residues specified by acidic side chains in many proteins, including growth factor receptors, transcription factors, and cytoskeletal proteins.[2,3] Aberrant expression and high CK2 kinase activity are characteristic of many cancers, promoting tumor survival and growth, and CK2 is a promising therapeutic target for malignant diseases.[4] CK2 exists in tetrameric complexes consisting of two catalytic subunits (CK2α and/or CK2α′) and two regulatory subunits (CK2β). The regulatory subunit is not essential for activity, but it confers specificity and can affect the ability of the catalytic subunits to phosphorylate certain substrates.[5] CK2 enhances the activity of several signaling pathways that are essential for cell proliferation and differentiation, including the nuclear factor-κB (NF-κB), phosphatidylinositol-3-kinase/AKT/mammalian target of rapamycin (PI3K/AKT/mTOR), and Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathways.[1,6,7] CK2 directly phosphorylates NF-κB p65 and IκB to enhance NF-κB signaling, and phosphorylates AKT to activate the mTOR pathway.[8] Our previous studies provided the first evidence that CK2 is critical for activation of the JAK/STAT signaling pathway in tumor cells and T cells.[7–10]

Inflammatory bowel diseases (IBDs) are chronic relapsing inflammatory disorders of the gastrointestinal tract and can be classified into two major subtypes, Crohn's disease (CD) and ulcerative colitis (UC).[11,12] It is widely accepted that IBDs are triggered by an inappropriate immune response, primarily by CD4$^+$ T cells to antigens of commensal gut bacteria in genetically susceptible cohorts.[13] In CD, there is a bias toward the production of proinflammatory cytokines associated with T helper type 1 (Th1) (interferon-γ [IFN-γ]) and Th17 (interleukin-17 [IL-17]) cells,[12,14] whereas UC is thought to be associated with Th2 cells producing IL-5 and IL-13.[15,16] Accordingly, one of the main therapeutic strategies for IBDs is to target CD4$^+$ T cells.

Growing evidence suggests that CK2 can modulate the function of immune cells, including CD4$^+$ T cells.[9,10,17–19] Historically, CK2 was thought to be constitutively expressed and active;[1] however, we recently demonstrated that CK2 protein and kinase activity are induced in CD4$^+$ T cells upon T cell receptor (TCR) stimulation.[9] Interestingly, Ulges et al.[19] and our group described the function of CK2 in regulating the Th17/T-regulatory cell (Treg) axis.[9] Utilizing CX-4945, a CK2α- and CK2α′-specific small-molecule inhibitor, small interfering RNA (siRNA) knockdown of CK2α, as well as genetic deletion of CK2α in CD4$^+$ T cells, our group demonstrated that CK2 activity promotes Th17 cell differentiation and inhibits generation of Foxp3$^+$ Treg cells.[9,10] Mechanistically, we determined that CK2α promotes Th17 cell differentiation and suppresses Tregs through the negative regulation of the transcription factor FoxO1.[10] Ulges et al.[19] demonstrated that T cell-specific deletion of CK2β also results in defective Th17 development and enhanced Treg generation. Taken together, these results suggest that both the catalytic activity conferred by CK2α and CK2α′ and CK2β-mediated regulatory mechanisms are important for Th17-promoting signaling pathways during CD4$^+$ T cell activation and lineage commitment. Importantly, targeting of CK2 systemically with pharmacological inhibition or by CD4$^+$ T

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cell-specific deletion of either CK2α or CK2β resulted in significant protection in a preclinical model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), which was associated with decreased Th17 cells and increased Tregs.[9,10,19] CK2β is also involved in the suppressive function of $CD4^+$ $Foxp3^+$ Tregs against allergy-promoting Th2 cells.[18] In addition, CK2 is critical for monocyte-derived dendritic cells to mature and produce cytokines to polarize effector T cells in response to chemicals related to allergic contact dermatitis.[17] Thus, CK2 appears to have important roles in regulating both innate and adaptive immune responses.[20] CK2 expression and activity is enhanced in epithelial cells during murine and human intestinal inflammation, and it was suggested that CK2 promotes mucosal homeostasis in colitis.[21] However, the exact function of CK2 in T cells during colitis is unknown and is the focus of this study.

We investigated the role of CK2α in $CD4^+$ T cells during chronic intestinal inflammation. We demonstrate that CK2α expression in $CD4^+$ T cells is critical for the induction of colitis. Genetic abrogation of CK2α in $CD4^+$ T cells led to diminished Th1 and Th17 cell differentiation in both the spleen and colon in the T cell transfer colitis model. We also demonstrated that CK2α is essential for normal $CD4^+$ T cell proliferation and controls $CD4^+$ T cell accumulation and responses in the spleen and colon through a cell-intrinsic manner. Mechanistically, CK2α regulates IL-12/STAT4 pathway activation by controlling IL-12 receptor (IL-12R) expression, which then promotes Th1 cell differentiation. In addition, CK2α controls expression of the transcription factor BATF (basic leucine zipper transcription factor, ATF-like) and costimulatory molecules CD40L (CD40 ligand) and ICOS (inducible costimulatory molecule) by regulating expression of NFAT2, a transcription factor vital for T cell activation and proliferation. Thus, our data demonstrate that CK2α contributes to the pathogenesis of colitis by promoting $CD4^+$ T cell proliferation and Th1 and Th17 responses.

RESULTS

CK2α expression and kinase activity in $CD4^+$ T cells is required for colitis

To assess the role of CK2α in T cells in the development of colitis, $CK2\alpha^{fl/fl}$dLck-Cre mice, in which CK2α is specifically deleted in T cells, were used.[10] We previously determined that $CD4^+$ T cells lacking CK2α, the major catalytic subunit of CK2, exhibit a significant decrease in overall CK2 kinase activity.[10] We transferred $CD45RB^{hi}$ $CD4^+$ T cells isolated from $CK2\alpha^{fl/fl}$ or $CK2\alpha^{fl/fl}$dLck-Cre mice into lymphopenic $Rag1^{-/-}$ mice with T cell and B cell deficiency to induce colitis.[22] $CD4^+$ T cells from $CK2\alpha^{fl/fl}$ mice induced severe colitis, as assessed by body weight loss, colon length, and weight and inflammation score (Fig. 1a–e). In contrast, $CD4^+$ T cells from $CK2\alpha^{fl/fl}$dLck-Cre mice induced only mild intestinal inflammation in $Rag1^{-/-}$ mice, as documented by lack of body weight loss (Fig. 1a), longer colon length (Fig. 1b), and lighter colon weight (Fig. 1c). Consistent with these findings, histopathological analysis showed less severe inflammation in the colon of $Rag1^{-/-}$ mice that received $CD4^+$ T cells from $CK2\alpha^{fl/fl}$dLck-Cre mice compared to $CD4^+$ T cells from $CK2\alpha^{fl/fl}$ mice (Fig. 1d, e). Serum concentrations of proinflammatory cytokines and chemokines such as IFN-γ, tumor necrosis factor-α (TNF-α), IL-12p40, chemokine (C-C motif) ligand 5, C-X-C motif chemokine ligand 9 (CXCL9), and CXCL10 were analyzed, and there were significantly lower concentrations of proinflammatory cytokines and chemokines in $Rag1^{-/-}$ mice that received $CD4^+$ T cells from $CK2\alpha^{fl/fl}$ dLck-Cre mice compared to $CK2\alpha^{fl/fl}$ mice (Fig. 1f). To exclude the possibility of genotoxicity of dLck-Cre, we transferred naive $CD4^+$ T cells from dLck-Cre mice into $Rag^{-/-}$ mice. $CD4^+$ T cells from dLck-Cre mice induced severe body weight loss compared to $CD4^+$ T cells from $CK2\alpha^{fl/fl}$dLck-Cre mice, and were comparable to $CD4^+$ T cells from $CK2\alpha^{fl/fl}$ mice (Fig. 1a). These results indicate that CK2α expression in $CD4^+$ T cells is required for development of colitis in this T cell adoptive transfer model.

CK2α controls T cell accumulation and cytokine responses in the spleen and colon

The T cell adoptive transfer colitis model is associated with the accumulation of $CD4^+$ Th1 and Th17 cells in the intestine.[22] To analyze the direct effects of CK2α on $CD4^+$ T cell responses, we isolated cells from the spleen and colon of $Rag1^{-/-}$ mice that received $CD4^+$ T cells from $CK2\alpha^{fl/fl}$ or $CK2\alpha^{fl/fl}$dLck-Cre mice and analyzed $CD4^+$ T cell accumulation and Th cell responses at 7 weeks after transfer. There were significantly fewer total mononuclear cells (Fig. 2a) and $CD4^+$ T cells (Fig. 2b) in both the spleen and colon from $Rag1^{-/-}$ recipients of CK2α-deficient $CD4^+$ T cells compared to the control group. Furthermore, we performed intracellular flow cytometric analysis of IL-17A and IFN-γ expression in $CD4^+$ T cells. Three distinct populations were detected in $Rag1^{-/-}$ recipients of $CD4^+$ T cells: IFN-γ^+, IL-$17A^+$, and double-positive IFN-γ^+IL-$17A^+$ cells (Fig. 2c). We observed significant reductions in the percentage of IFN-γ^+ and IFN-γ^+IL-$17A^+$ $CD4^+$ T cells in the spleen and colon from $Rag1^{-/-}$ recipients of CK2α-deficient $CD4^+$ T cells (Fig. 2d). There were significant reductions in the absolute cell numbers of IFN-γ^+ $CD4^+$ T cells in the spleen and colon, and a significant reduction in the absolute cell numbers of IL-$17A^+$ and IFN-γ^+IL-$17A^+$ $CD4^+$ T cells in the colon (Fig. 2e). These findings indicate that CK2α controls both Th1 and Th17 cell differentiation in vivo. Collectively, these data suggest that CK2α regulates the accumulation, differentiation, and colitogenic properties of $CD4^+$ T cells in the T cell adoptive transfer colitis model.

CK2α controls Th1 differentiation through the IL-12/STAT4 pathway

We previously determined that CK2α is critical for Th17 cell differentiation in the EAE model, but that Th1 cell differentiation was not affected.[9,10] However, a decreased frequency of Th1 IFN-γ^+ $CD4^+$ T cells was found in the spleen and colon from $Rag1^{-/-}$ recipients of CK2α-deficient $CD4^+$ T cells (Fig. 2d), suggesting that in the colitis model, CK2α may influence Th1 cell differentiation. As such, we next examined how CK2α may regulate the differentiation of Th1 cells. CK2α-deficient $CD4^+$ T cells exhibited a defect in polarization to the Th1 phenotype in vitro, as IFN-γ production by CK2α-deficient $CD4^+$ T cells was significantly reduced (Fig. 3a, b). IL-12 signaling through STAT4 is essential for induction of IFN-γ production and commitment of Th1 cells.[23,24] To determine the effect of CK2α on the activation of STAT4, naive $CD4^+$ T cells were activated for 24 h, and then stimulated with IL-12 for the indicated time points. Robust STAT4 phosphorylation in response to IL-12 was detected in $CD4^+$ T cells from $CK2\alpha^{fl/fl}$ mice, which was diminished in CK2α-deficient $CD4^+$ T cells (Fig. 3c, d). IL-12 signals through IL-12R to induce STAT4 activation.[25] We next examined expression levels of both IL-12Rβ1 and IL-12Rβ2 in $CD4^+$ T cells. CK2α-deficient $CD4^+$ T cells expressed significantly lower levels of IL-12Rβ1 and IL-12Rβ2 than CK2α-sufficient $CD4^+$ T cells upon TCR activation (Fig. 3e, f). Thus, in the absence of CK2α, decreased IL-12R expression results in reduced IL-12-induced STAT4 phosphorylation, thereby resulting in impaired Th1 cell differentiation.

CK2α is essential for $CD4^+$ T cell proliferation but not survival in vitro and in vivo

The failure of CK2α-deficient $CD4^+$ T cells to accumulate in the spleen and colon (Fig. 2a, b) may reflect a failure to proliferate and/or survive in these organs. To address these possibilities, we assessed the proliferation capacity of CK2α-sufficient and CK2α-deficient $CD4^+$ T cells both in vitro and in vivo. Carboxyfluorescein succinimidyl ester (CFSE)-labeled $CD4^+$ T cells were stimulated with graded concentrations of anti-CD3 and anti-CD28 antibodies (Abs), and proliferation of $CD4^+$ T cells was assessed by CFSE

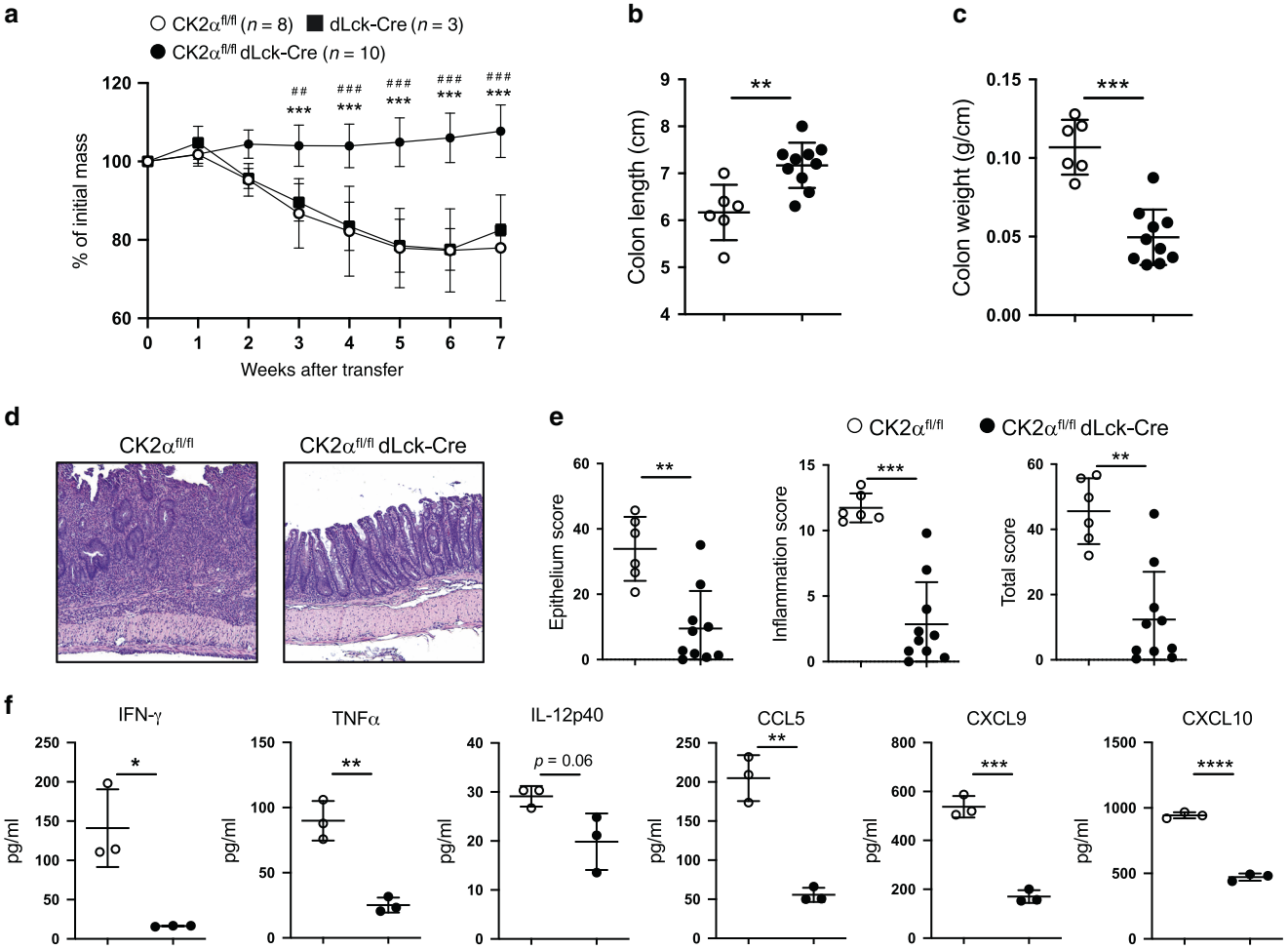

Fig. 1 CK2α expression in CD4$^+$ T cells is required for colitis. C57BL/6 Rag1$^{-/-}$ mice (male or female) were injected i.v. with 1×10^6 naive CD4$^+$ T cells from sex- and age-matched CK2α$^{fl/fl}$, dLck-Cre, or CK2α$^{fl/fl}$dLck-Cre mice at the age of 8–10 weeks. Body weight of recipients was monitored weekly. Seven weeks after transfer, mice were sacrificed and assessed for intestinal inflammation. **a** Weight change of recipient mice. CK2α$^{fl/fl}$, $n = 8$; dLck-Cre, $n = 3$; CK2α$^{fl/fl}$dLck-Cre, $n = 10$. *CK2α$^{fl/fl}$ compared to CK2α$^{fl/fl}$dLck-Cre; #dLck-Cre compared to CK2α$^{fl/fl}$dLck-Cre. **b** Colon length and **c** colon weight. Each dot represents one mouse. **d** Representative pictures of recipient colon sections stained with H&E. **e** Pathological scores of colons in recipient mice. **f** Cytokines and chemokines in serum were detected by multiplex ELISA. Data represent pooled results from three independent experiments. CK2α$^{fl/fl}$, $n = 6$; CK2α$^{fl/fl}$dLck-Cre, $n = 10$. Bars represent the mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ##$p < 0.01$, and ###$p < 0.001$.

dilution. CK2α-deficient T cells exhibited reduced proliferation compared to CD4$^+$ T cells from CK2α$^{fl/fl}$ mice (Fig. 4a, b). We also analyzed the survival of CK2α-sufficient and CK2α-deficient CD4$^+$ T cells by Annexin V staining, and no differences were observed (Supplementary Fig. 1A, B). We next transferred CFSE-labeled CK2α-sufficient or CK2α-deficient CD4$^+$ T cells into Rag1$^{-/-}$ mice to analyze their proliferative ability in vivo. There was significantly less proliferation of CK2α-deficient CD4$^+$ T cells compared with CD4$^+$ T cells from CK2α$^{fl/fl}$ mice in the spleen (Fig. 4c, d), but no differences in Annexin V$^+$ CD4$^+$ T cells between the two groups were observed (Supplementary Fig. 1C, D). Altogether, these findings indicate that CK2α controls T cell accumulation by affecting cell proliferation but not survival.

CK2α controls T cell accumulation in the spleen and colon through a cell-intrinsic manner

To further address whether CK2α affects CD4$^+$ T cell function through cell-intrinsic or cell-extrinsic mechanisms, we took advantage of a co-transfer model.[26] We administered equal numbers of congenic CD45.1 C57BL/6- and CD45.2 CK2α$^{fl/fl}$dLck-Cre-naive CD4$^+$ T cells into Rag1$^{-/-}$ recipients. As a control, we transferred a 1:1 ratio of CD45.1 C57BL/6- and CD45.2 CK2α$^{fl/fl}$-naive CD4$^+$ T cells into Rag1$^{-/-}$ recipients. Two weeks after transfer, the ratio between CK2α-sufficient CD4$^+$ T cells to C57BL/6 CD4$^+$ T cells in the spleen, mesenteric lymph nodes (MLNs), and colon lamina propria was slightly lower compared to the ratio right before cell transfer (Fig. 5a, c). However, the relative ratio was significantly higher than the ratio between CD4$^+$ T cells from CK2α-deficient mice to congenic C57BL/6 CD4$^+$ T cells (Fig. 5b, c). The relative ratio was consistent in the spleen, MLNs, and colon lamina propria in both groups (Fig. 5a–c), and remained constant 4 weeks after transfer in both groups, indicating that there is no proliferation defect of CK2α-sufficient CD4$^+$ T cells compared to C57BL/6 CD4$^+$ T cells (Supplementary Fig. 2). CD4$^+$ T cell responses in the spleen and colon in the co-transfer system were analyzed. We observed that the percentages of IFN-γ$^+$- and IFN-γ$^+$IL-17A$^+$-producing cells in CK2α-deficient CD4$^+$ T cells were significantly lower compared to their C57BL/6 counterparts in the spleen and colon (Fig. 5d, e). There were no differences in CD4$^+$ T cell responses between C57BL/6 and CK2α$^{fl/fl}$ T cells, as shown by comparable percentages of IFN-γ$^+$ and IFN-γ$^+$IL-17A$^+$ CD4$^+$ T cells between C57BL/6 and CK2α$^{fl/fl}$ T cells (Supplementary Fig. 3).

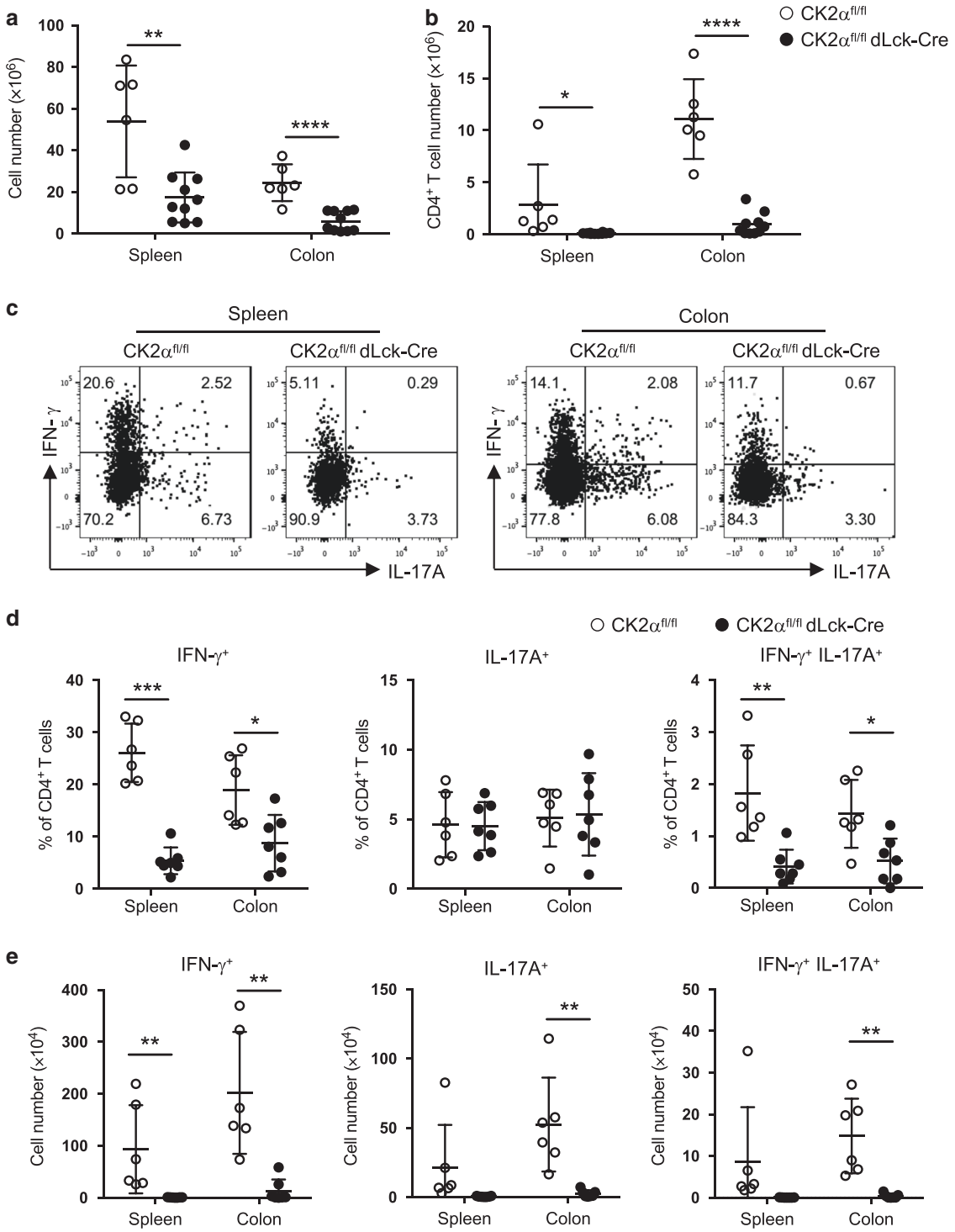

Fig. 2 CK2α controls T cell accumulation in the spleen and colon. Absolute number of mononuclear cells (**a**) and $CD4^+$ T cells (**b**) in the spleen and colon were detected at 7 weeks after transfer. **c** Representative flow cytometry profile of IL-17A and IFN-γ production by $CD4^+$ T cells in the spleen and colon. **d** Percentages and **e** absolute cell numbers of IFN-γ^+, IL-$17A^+$, and IFN-γ^+IL-$17A^+$ $CD4^+$ T cells in the spleen and colon is shown. Data represent pooled results from three independent experiments. $CK2\alpha^{fl/fl}$, $n = 6$; $CK2\alpha^{fl/fl}$dLck-Cre, $n = 7$. Bars represent the mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

These results demonstrate that the requirement of cell-intrinsic CK2α signaling for T cell accumulation in the intestine cannot be rescued by the presence of CK2α-sufficient T cells and their subsequent downstream inflammatory signals. Therefore, CK2α activity in $CD4^+$ T cells is required for their effective accumulation through a cell-intrinsic manner.

CK2α controls $CD4^+$ T cell responses, in part, by regulating expression of NFAT2

To understand the molecular mechanisms that confer CK2α function in $CD4^+$ T cells in the development of colitis, $CD4^+$ T cells were isolated from the colon 6 weeks after transfer and subjected to RNA-sequencing (RNA-seq). A total of 122 genes were

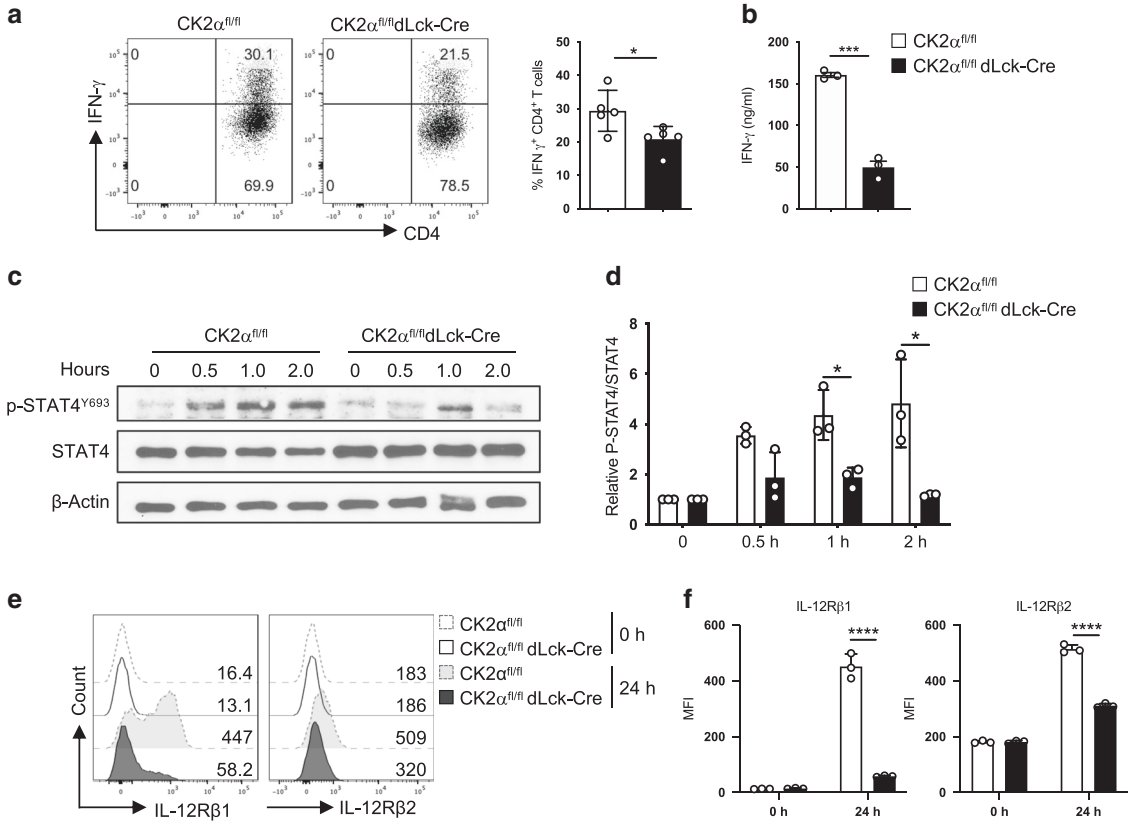

Fig. 3 CK2α controls Th1 differentiation through the IL-12/STAT4 pathway. a Naive CD4$^+$ T cells from CK2α$^{fl/fl}$ or CK2α$^{fl/fl}$dLck-Cre mice were polarized under Th1 conditions [anti-CD3 (1 μg/ml), anti-CD28 (1 μg/ml), IL-12 (10 ng/ml), and anti-IL-4 (10 μg/ml)] for 3 days. IFN-γ production by CD4$^+$ T cells was detected by intracellular staining. Representative flow cytometry profiles of IFN-γ production by CD4$^+$ T cells is shown ($n = 5$). **b** IFN-γ in the supernatant was measured by ELISA ($n = 3$). **c** Naive CD4$^+$ T cells from CK2α$^{fl/fl}$ mice or CK2α$^{fl/fl}$dLck-Cre mice were activated with anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) for 24 h, stimulated with IL-12 (10 ng/ml) for the indicated time points, and then phosphorylated P-STAT4 Y693 in CD4$^+$ T cells was detected by immunoblotting. **d** Relative ratio of phosphorylated P-STAT4 Y693 to total STAT4 at the indicated time points is shown ($n = 3$). **e** Naive CD4$^+$ T cells from CK2α$^{fl/fl}$ or CK2α$^{fl/fl}$dLck-Cre mice were activated with anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) for 24 h, and expression levels of IL-12Rβ1 and IL-12Rβ2 detected by flow cytometry. Representative line graphs are shown. **f** Quantitation of MFI of IL-12Rβ1 and IL-12Rβ2 expression is shown. $n = 3$ in each group. Bars represent the mean ± SD. *$P < 0.05$, ***$p < 0.001$, and ****$p < 0.0001$.

significantly increased and 132 genes were significantly decreased in CK2α-deficient CD4$^+$ T cells compared to CK2α-sufficient CD4$^+$ T cells (Fig. 6a). Gene set enrichment analysis (GSEA) of the data revealed that differentially expressed genes were associated with the TCR signaling pathway (Fig. 6b). CK2α-deficient CD4$^+$ T cells express lower levels of genes associated with TCR and co-stimulatory signaling pathways, such as *Nfatc1*, *Batf*, *Icos*, and *Cd40lg* (Fig. 6c), which are important for T cell activation and proliferation.[27–30] We validated RNA-sequencing data by analyzing gene expression levels in CD4$^+$ T cells from Rag1$^{-/-}$ mice with colitis by RT-PCR, and demonstrated significantly lower expression levels of *Nfatc1*, *Icos*, *Cd40lg*, and *Batf* in CD4$^+$ T cells from CK2α$^{fl/fl}$dLck-Cre mice compared to CK2α$^{fl/fl}$ mice (Fig. 6d). Studies have shown that CD40L and ICOS are NFAT-dependent molecules and the transcription factor BATF is also regulated by NFAT in T follicular helper (Tfh) cells.[31–34] To determine a possible role of NFAT2 in gene expression in CD4$^+$ T cells in vitro, CD4$^+$ T cells from CK2α$^{fl/fl}$ and CK2α$^{fl/fl}$dLck-Cre mice were stimulated with anti-CD3 and anti-CD28 Abs for 24 h, and expression levels of NFAT2 were analyzed by flow cytometry. CD4$^+$ T cells from CK2α$^{fl/fl}$dLck-Cre mice expressed significantly lower levels of NFAT2 after TCR stimulation compared to CD4$^+$ T cells from CK2α$^{fl/fl}$ mice (Fig. 6e). Next, we sought to determine whether the defect in CD4$^+$ T cell function in CK2α$^{fl/fl}$dLck-Cre mice was due to decreased NFAT2 expression. To do this, a constitutively active form of NFAT2 (caNFAT2)[31] was overexpressed in CK2α$^{fl/fl}$dLck-Cre CD4$^+$ T cells in vitro. CK2α-deficient CD4$^+$ T cells expressed lower levels of ICOS, CD40L, and BATF compared to CK2α-sufficient CD4$^+$ T cells, and caNFAT2 rescued expression levels of ICOS, and partially rescued CD40L and BATF levels (Fig. 6f, g). Importantly, caNFAT2 also partially rescued the defect of CD4$^+$ T cell proliferation in CK2α$^{fl/fl}$dLck-Cre mice, as assessed by Ki-67 staining (Fig. 6h, i). Altogether, these data demonstrate that CK2α controls CD4$^+$ T cell function, in part, by regulating NFAT2 expression.

DISCUSSION

The majority of our current knowledge on CK2 comes from extensive studies in the context of cancer.[35] However, growing evidence suggests the crucial involvement of CK2 in both innate and adaptive immune responses.[20] In this study, we demonstrate that CK2α expression and kinase activity is required for CD4$^+$ T cells to induce colitis. We found that CK2α controls CD4$^+$ T cell proliferation and differentiation into Th1 and Th17 cells, which then consequently affects the pathogenesis of T cell-mediated colitis.

Studies show that there is a bias toward the production of proinflammatory cytokines associated with Th1 and Th17 cells in patients with CD.[12,14] We took advantage of the T cell adoptive transfer colitis model, which is associated with the accumulation of CD4$^+$ Th1 and Th17 cells in the intestine,[22] to elucidate the

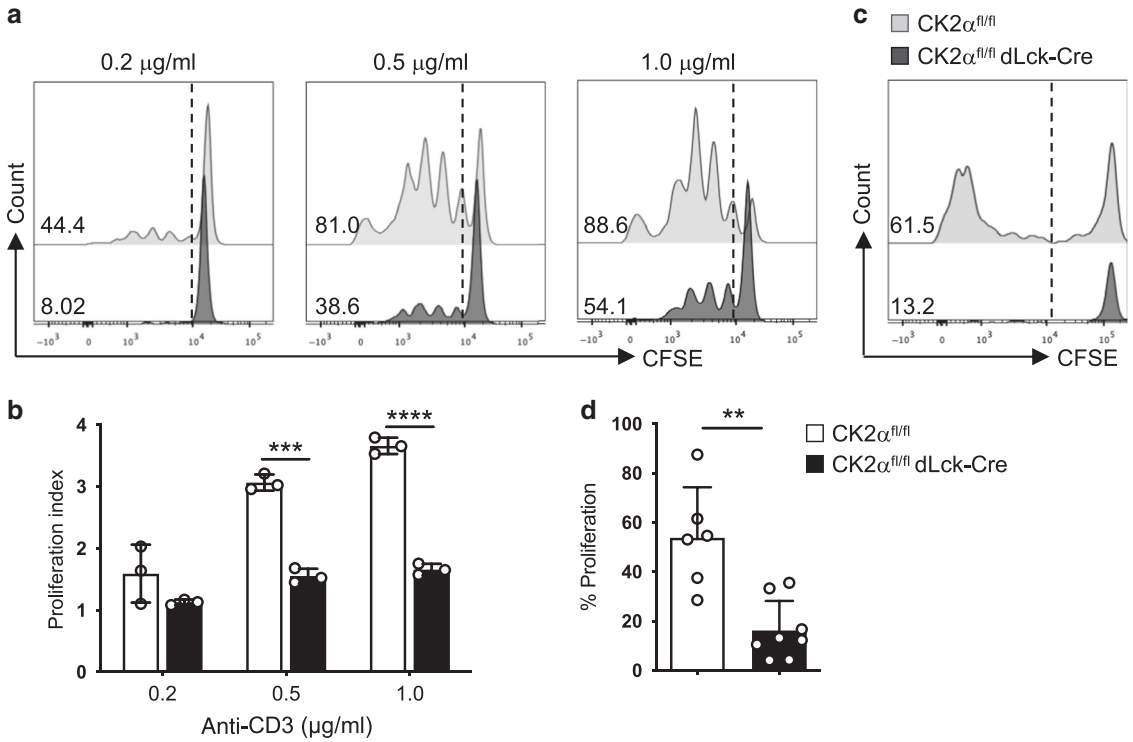

Fig. 4 CK2α is essential for CD4$^+$ T cell proliferation in vitro and in vivo. a CFSE-labeled naive CD4$^+$ T cells were activated with the indicated concentrations of anti-CD3 (0.2, 0.5, and 1.0 μg/ml) and 1.0 μg/ml of anti-CD28 Abs for 72 h, and proliferation was assessed by CFSE dilution. Representative line graphs are shown. **b** Proliferation index of CD4$^+$ T cells is shown ($n = 3$). **c** A total of 1×10^6 CFSE-labeled naive CD4$^+$ T cells from CK2α$^{fl/fl}$ or CK2α$^{fl/fl}$dLck-Cre mice were transferred into Rag1$^{-/-}$ mice by i.v. injection. Mice were sacrificed 5 days later, and proliferation of CD4$^+$ T cells from the spleen was assessed by CFSE dilution. Representative line graphs are shown. **d** Frequencies of cells undergoing proliferation is shown. Data represent pooled results from three independent experiments. CK2α$^{fl/fl}$, $n = 6$; CK2α$^{fl/fl}$dLck-Cre, $n = 8$. Bars represent the mean ± SD. $^{**}P < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

function of CK2α in CD4$^+$ T cells. Strikingly, we found that CK2α-deficient CD4$^+$ T cells failed to induce severe inflammation in the colon (Fig. 1), which was associated with decreased numbers of CD4$^+$ T cells and IFN-γ- and IL-17A-producing CD4$^+$ T cells both in the spleen and colon. These data indicate that CK2α controls Th1 and Th17 differentiation in the context of colitis. This is consistent with our previous studies, which showed that CK2α regulates Th17 differentiation in the EAE model.[9,10] However, there was no significant change in Th1 cell responses in the EAE model. Furthermore, in the EAE model, CK2α deficiency in CD4$^+$ T cells promoted the differentiation of Foxp3$^+$ Treg cells, which was not observed in the colitis model. Thus, the regulatory functions of CK2α in CD4$^+$ T cell differentiation may be different in distinct inflammatory disorders or target organs (central nervous system versus colon) due to microenvironmental differences. Second, the differential response of Th1 cells in vitro may be caused by the strength of TCR stimulation. In this study, we use lower concentrations of anti-CD3 (0.2–1.0 μg/ml), compared to our previous study (10 μg/ml),[10] to activate CD4$^+$ T cells in vitro to mimic the in vivo colitis condition, and found that CK2α-deficient CD4$^+$ T ells showed impaired Th1 differentiation (Fig. 3a). Collectively, in the T adoptive transfer colitis model, CK2α regulates the differentiation of both Th1 and Th17 cells.

Importantly, STAT4 is essential for Th1 differentiation and function, and is predominantly activated by IL-12 and to a lesser extent by IFNα and IL-23.[36,37] Genome-wide association studies (GWAS) have shown that the *STAT4* gene is significantly associated with both CD and UC,[38,39] and the IL-12–IL-23 signaling pathway is an attractive therapeutic target in these diseases.[40] In this study, we demonstrate that CK2 is critical for IL-12-induced phosphorylation of STAT4 (Fig. 3c) by directly controlling IL-12 receptor expression (Fig. 3e, f), thereby linking CK2α to the signaling pathway critical for Th1 cell differentiation. Our previous study showed that CK2 was a novel interaction partner of JAK1/2 and potentiated downstream STAT3 activation and gene expression.[7] Thus, there is the possibility that CK2α may directly affect JAK2/STAT4 activation through TCR-induced CK2α expression, and this will be addressed in future studies.

CK2 participates in diverse biological processes, including transcription, translation, cell growth, and proliferation in normal and disease states, particularly in cancers.[4] CK2 promotes cancer cell growth and proliferation, as well as suppresses cell apoptosis,[41] which are associated with worse prognosis. Here, we demonstrate that CK2α is critical for CD4$^+$ T cell proliferation, but has no effect on CD4$^+$ T cell survival, especially under lower doses of TCR stimulation (Fig. 4). This indicates that CK2 has cell-specific functions in cancer cells and immune cells. Considering that aberrant aggregated CD4$^+$ T cells in the intestine is one symptom in patients with IBD,[11,42] targeting CK2 in CD4$^+$ T cells to limit cell proliferation might be a therapeutic strategy in IBD.

Another striking finding from the RNA-seq data was that *NFAT2*, *ICOS*, *CD40L*, and *BATF* expression was significantly decreased in CK2α-deficient CD4$^+$ T cells isolated from the colon (Fig. 6). These genes are associated with the TCR signaling pathway. NFAT transcription factors are key regulators of T cell activation and exhaustion, and consist of five members, NFAT1, NFAT2, NFAT3, NFAT4, and NFAT5.[27] Three members of the NFAT family including NFAT1, NFAT2, and NFAT4, are present in T cells, and play vital roles in T cell development, activation, differentiation, and function.[27,43] During T cell activation, NFAT proteins are dephosphorylated by activated calcineurin, which leads to their nuclear translocation and induction of NFAT-mediated gene transcription.[44] NFAT1 binds to IFN-γ promoter regions to regulate Th1 cell responses.[45] NFAT1 and NFAT2 also directly bind to the IL-17

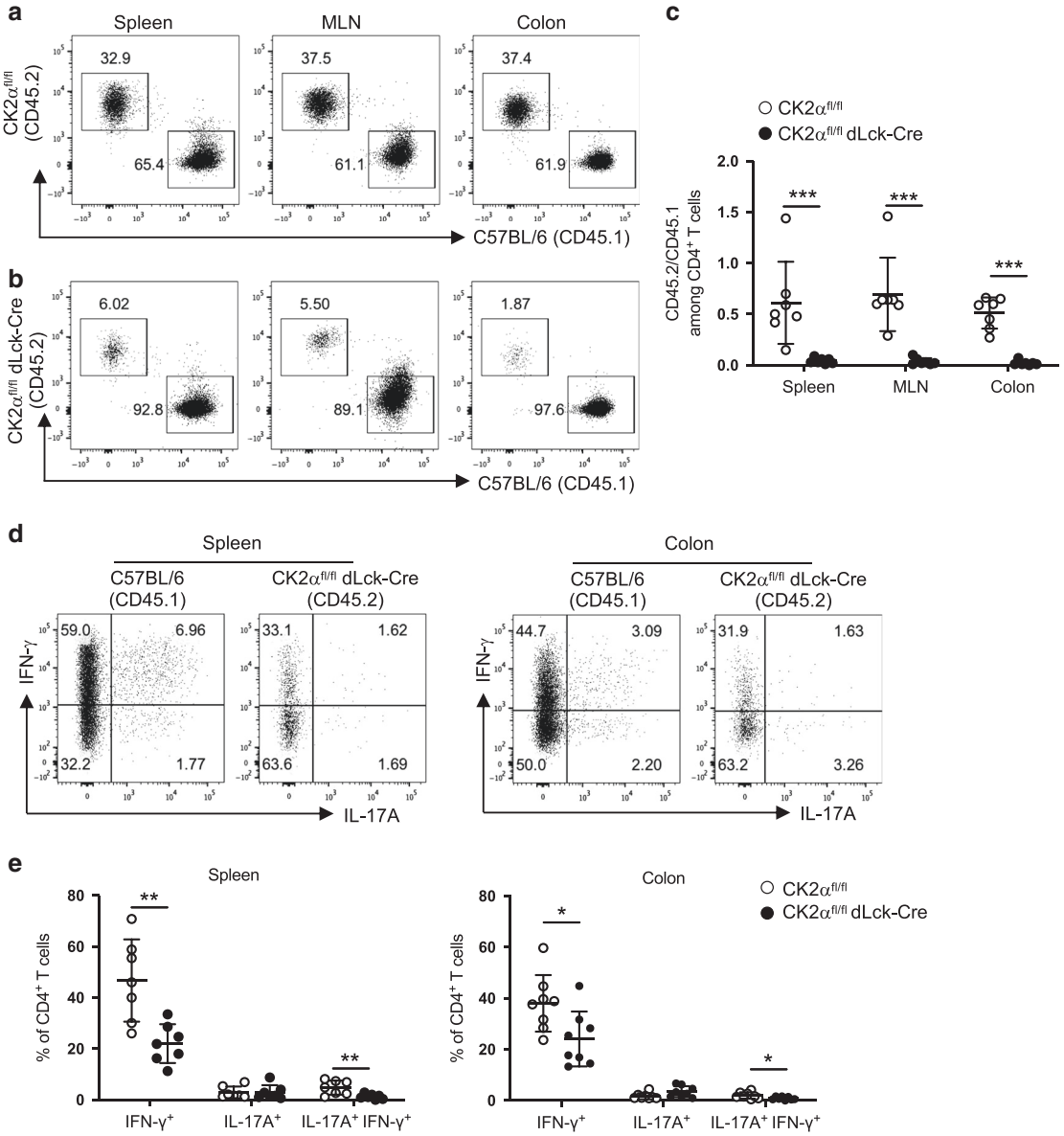

Fig. 5 CK2α controls T cell accumulation in the spleen and colon through a cell-intrinsic manner. Naive $CD4^+$ T cells from $CK2\alpha^{fl/fl}$ mice ($CD45.2^+$) or $CK2\alpha^{fl/fl}$dLck-Cre mice ($CD45.2^+$) were co-transferred with naive $CD4^+$ T cells from C57BL/6 mice ($CD45.1^+$) at a ratio of 1:1 (5×10^5: 5×10^5) into $Rag1^{-/-}$ mice by i.v. injection. Recipients were sacrificed 2 weeks after transfer, and the percentage of C57BL/6 $CD4^+$ T cells ($CD45.1^+$) and CK2α-sufficient or CK2α-deficient $CD4^+$ T cells ($CD45.2^+$) in the spleen, mesenteric lymph node (MLN), and colon was detected by flow cytometry. **a**, **b** Representative flow cytometry profile of $CD4^+$ T cells recovered from the indicated sites. **c** Ratio of CD45.2/CD45.1 $CD4^+$ T cells in the spleen, MLN, and colon. **d** Representative flow cytometry profiles of IFN-γ and IL-17A production by $CD4^+$ T cells derived from C57BL/6 and $CK2\alpha^{fl/fl}$dLck-Cre mice in the spleen and colon. **e** Frequencies of IFN-γ^+, IL-$17A^+$, and IL-$17A^+$IFN-γ^+ $CD4^+$ T cells derived from C57BL/6 and $CK2\alpha^{fl/fl}$dLck-Cre mice in the spleen and colon is shown. Data represent pooled results from three independent experiments. $CK2\alpha^{fl/fl}$, $n = 8$; $CK2\alpha^{fl/fl}$dLck-Cre, $n = 8$. Bars represent the mean ± SD. $^*P < 0.05$ and $^{***}p < 0.001$.

promoter region to regulate cytokine production.[46,47] Moreover, NFAT2 cooperates with NFAT1 to control the expression levels of ICOS, CXCR5, and CD40L, as well as control the expression of IRF4 and BATF in the differentiation of Tfh cells.[31–34] Interestingly, GWAS studies have shown that *NFAT2 and ICOS* are newly identified candidate genes in IBD susceptibility loci associated with modulation of T cell responses.[48] In addition, $CD40L^+$ T cells are increased in patients with IBD.[49] ICOS-ICOSL and CD40-CD40L are important costimulatory pathways to amplify immune responses and promote inflammation.[50] One study demonstrated that CK2 binds the N terminus of NFAT2 and phosphorylates important amino acid residues to regulate NFAT2 activity.[51] Our data show that CK2α can also regulate NFAT2 expression both at the messenger RNA and protein levels in $CD4^+$ T cells (Fig. 6d, e). More importantly, we demonstrated that overexpression of caNFAT2 in CK2α-deficient $CD4^+$ T cells partially rescued the defect in T cell proliferation and also partially rescued expression of the NFAT2-dependent molecules ICOS, CD40L, and BATF (Fig. 6f–i). These findings indicate that the CK2–NFAT2 axis is vital for regulating T cell activation, proliferation, and differentiation, and thereafter leads to aberrant $CD4^+$ T cell immune responses in T cell-induced colitis. The exact manner by which CK2α regulates NFAT2 expression is still unclear and under study. Important to note is that caNFAT2 did not completely rescue the $CD4^+$ T cell phenotype caused by CK2α deficiency. This raises the possibility that CK2α has other targets, not only NFAT2, which are essential

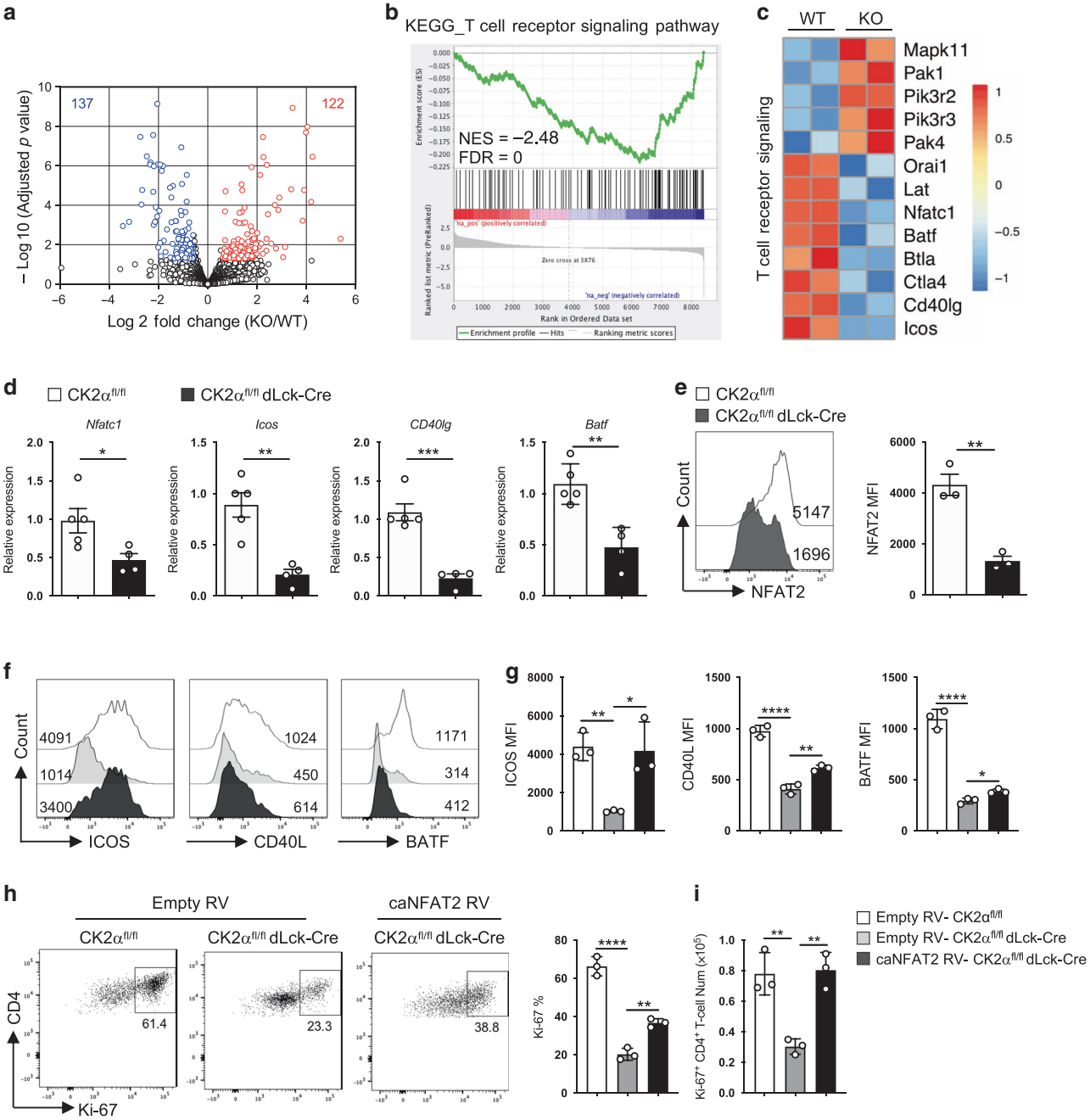

Fig. 6 CK2α controls CD4$^+$ T cell responses by regulating expression of NFAT2. RNA sequencing of CK2α$^{fl/fl}$ CD4$^+$ T cells (WT) and CK2α$^{fl/fl}$dLck-Cre CD4$^+$ T cells (KO) from the colon at 6 weeks after colitis induction was performed (three to five mice were combined per experiment, two experiments in each group). **a** Summary of genes differentially regulated by CK2α using the following cutoffs are shown: $p < 0.05$, fold change >1.5. **b** GSEA plot shows enrichment of RNA-sequencing data compared with KEGG pathway dataset. **c** Heat map shows TCR signaling relative gene expression. **d** Selected gene expression was validated in CD4$^+$ T cells from colitis Rag1$^{-/-}$ mice by qRT-PCR. CK2α$^{fl/fl}$, $n = 5$; CK2α$^{fl/fl}$dLck-Cre, $n = 4$. **e** Naive CD4$^+$ T cells from CK2α$^{fl/fl}$ and CK2α$^{fl/fl}$dLck-Cre mice were activated with 0.5 μg/ml of anti-CD3 and 1.0 μg/ml of anti-CD28 Abs for 24 h, and the expression of NFAT2 was detected by flow cytometry. Representative line graphs and quantitation are shown. $n = 3$ in each group. **f** Naive CD4$^+$ T cells from CK2α$^{fl/fl}$ and CK2α$^{fl/fl}$dLck-Cre mice were activated and transduced with caNFAT2 or empty control vector. GFP$^+$-transduced cells were sorted and stimulated with 0.1 μg/ml of anti-CD3 and 1.0 μg/ml of anti-CD28 Abs for 48 h, and the expression of ICOS, CD40L, and BATF was detected by flow cytometry. Representative line graphs are shown. **g** Quantitation of relative MFI of ICOS, CD40L, and BATF is shown. $n = 3$ in each group. **h** GFP$^+$ cells were sorted and stimulated with 0.1 μg/ml of anti-CD3 and 1.0 μg/ml of anti-CD28 Abs for 48 h, and proliferating cells were detected by Ki-67 staining. Representative line graphs and quantitation are shown. **i** Quantitation of absolute number of Ki-67$^+$ CD4$^+$ T cells is shown $n = 3$ in each group. Bars represent the mean ± SD. *$P < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

for CD4$^+$ T cell responses. These are important questions to be addressed in future studies.

Taken together, our data reinforce the critical function of CK2α in CD4$^+$ T cells. We demonstrated that CK2α is vital for CD4$^+$ T cell colitogenic properties, regarding aspects of regulating Th1 and Th17 cell differentiation and cell proliferation. Mechanistically, CK2α controls STAT4 activation by controlling IL-12R expression in CD4$^+$ T cells to regulate Th1

cell differentiation, and regulates NFAT2 expression to influence CD4+ T cell function upon T cell activation. These findings suggest that CK2α may be a therapeutic target in IBD and potentially in other T cell-mediated diseases.

METHODS

Mice

CK2α$^{fl/fl}$dLck-Cre mice,[10] CK2α$^{fl/fl}$ mice, dLck-Cre mice,[10] Rag1$^{-/-}$ mice, and CD45.1 C57BL/6 mice were bred and maintained under specific pathogen-free conditions in the animal facility at the University of Alabama at Birmingham (UAB). Male and female mice between 8 and 12 weeks were used for all experiments. All experimental procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of UAB.

CD4+ T cell purification

For in vivo transfer, total CD4+ T cells were first purified from the spleen and peripheral lymph nodes using the Dynabeads™ CD4 Positive Isolation Kit (Invitrogen, Carlsbad, CA), and then naive CD4+ T cells were sorted as CD4+CD25−CD45RB^{hi} on an Aria II (BD Bioscience, San Jose, CA), routinely to 98% purity. For in vitro activation and polarization, naive CD4+ T cells were enriched from the spleen and peripheral lymph nodes by the EasySep™ Mouse Naive CD4+ T Cell Kit (STEMCELL Technologies Inc., Vancouver, BC), routinely to 90–95% purity.

T cell transfer model of colitis

Naive CD4+CD25−CD45RB^{hi} T cells from CK2α$^{fl/fl}$, dLck-Cre, or CK2α$^{fl/fl}$dLck-Cre mice were transferred via intravenous (i.v.) tail injection to sex- and age-matched Rag1$^{-/-}$ mice (1×10^6 cells per mouse).[22] Weight loss of recipient Rag1$^{-/-}$ mice was monitored weekly, and mice were sacrificed 6–7 weeks after transfer. Samples of proximal, middle, and distal colon were fixed in 10% formalin. Paraffin-embedded samples were cut into 6 µm sections, followed by hematoxylin and eosin staining, and histopathology was evaluated in a double-blinded manner by a veterinary pathologist. Epithelium damage and inflammation severity were scored separately and a total score was given accordingly. Briefly, epithelium damage was evaluated in all sections by hyperplasia, goblet cell loss, degeneration and necrosis, ulceration, and dysplasia. Inflammation severity was evaluated by crypt exudate, lamina propria and submucosal inflammatory cell accumulation intensity, submucosal edema distribution, and transmural inflammation. Severity of lesions were graded as follows: 0 = normal; 1 = mild; 2 = moderate; 3 = severe.[52,53]

In co-transfer experiments, naive CD4+CD25−CD45RB^{hi} T cells from CK2α$^{fl/fl}$ or CK2α$^{fl/fl}$dLck-Cre mice were co-transferred with naive CD4+CD25−CD45RB^{hi} T cells from CD45.1 C57BL/6 mice at the ratio of 1:1 (5×10^5:5×10^5) to sex- and age-matched Rag1$^{-/-}$ mice. Recipient Rag1$^{-/-}$ mice were sacrificed 2 and 4 weeks after transfer to evaluate CD4+ T cell accumulation and responses in the spleen and colon.

Lymphocyte preparation

Single-cell suspensions of spleen and lymph nodes were prepared as previously described[9,10] and resuspended in R10 medium (RPMI 1640 with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES, 1 mM sodium pyruvate, and 50 µM β-mercaptoethanol). Colon lamina propria lymphocytes were prepared using the Lamina Propria Dissociation Kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Cells were then isolated using a 40/75% discontinuous Percoll gradient, washed, and resuspended in R10 medium.

Flow cytometry

Cell surface staining and intracellular staining was performed as previously described.[10] For cytokine production analysis, cells were stimulated with 50 ng/ml of phorbol-12-myristate-13-acetate (Sigma-Aldrich, St. Louis, MO) and 750 µg/ml of ionomycin (Sigma-Aldrich, St. Louis, MO) in the presence of GolgiStop (BD Biosciences, San Jose, CA) for 4 h. After staining of cell surface molecules, cells were fixed and permeabilized using the Foxp3 Staining Buffer Set (eBioscience, Grand Island, NY).[9] Stained cells were run on an LSRII flow cytometer (BD Biosciences, San Jose, CA), and data were analyzed using the FlowJo software (Tree Star, Inc, Ashland, OR). The following Abs were used in this study (all BioLegend except where noted otherwise): anti-CD3ε PerCP-Cy5.5/PE-Cy7/APC (clone 145-2C11); anti-CD4 Pacific Blue/FITC/Alexa Fluor 647/APC-Cy7/ PE (clone RM4-5); anti-CD11b FITC (clone M1-70); anti-CD11b BUV395 (clone M1-70, BD Bioscience), anti-CD45 APC-Cy7 (clone 30-F11); anti-CD45.1 Alexa Fluor 488/PerCP-Cy5.5 (clone A20); anti-CD45.2 Alexa 647/APC-Cy7 (clone 104); anti-CD45RB FITC (clone C363.16A), anti-CD25 Alexa Fluor 647/PE-Cy7 (clone PC61.5); anti-IFN-γ Pacific Blue/PE-Cy7 (clone XMG1.2); anti-IL-17A APC (clone TC11-18H10); anti-Ki-67 Brilliant Violet 421 (clone 16A8); anti-CD40L PE-Cy7 (clone 24-31); anti-ICOS APC (clone 15F9); anti-BATF PE (clone S39-1060, BD Bioscience); NFAT2 (Cell Signaling Technology); Alexa Fluor 647 anti-rabbit IgG (H + L) (Jackson Immuno Research Labs); anti-CD212 (IL-12Rβ1) PE (Clone 114, BD Bioscience); and anti-IL-12Rβ2 Alexa Fluor 488 (R&D System).

Naive CD4+ T cell activation and polarization

For in vitro activation, naive CD4+ T cells were cultured in R10 medium and stimulated with plate-bound anti-CD3 (0.2, 0.5, 1.0 µg/ml) (clone 145-2C11, BioX Cell, West Lebanon, NH) and soluble anti-CD28 Abs (1.0 µg/ml) (clone 37.51, BioX Cell, West Lebanon, NH) for 24 and 48 h. For Th1 cell polarization, cells were stimulated with plate-bound anti-CD3 (1.0 µg/ml) and soluble anti-CD28 Abs (1.0 µg/ml) in the presence of IL-12 (10 ng/ml, BioLegend) and anti-IL-4 Ab (10 µg/ml) (clone 11B11, BioX Cell, West Lebanon, NH) for 72 h. For phospho-STAT4 detection, cells were activated with plate-bound anti-CD3 (1.0 µg/ml) and soluble anti-CD28 (1.0 µg/ml) for 24 h, and then stimulated with IL-12 (10 ng/ml) for the indicated time points.

Proliferation assays

For the in vitro proliferation assay, naive CD4+ T cells were incubated with 5 µM CFSE, washed, and activated with plate-bound anti-CD3 (0.2, 0.5, and 1.0 µg/ml) and soluble CD28 Abs (1.0 µg/ml) for 72 h. CFSE dilution was detected by flow cytometry, and proliferation index was calculated by FlowJo. For the in vivo proliferation assay, naive CD4+CD25−CD45RB^{hi} T cells were labeled with 5 µM CFSE, and then 1×10^6 CFSE-labeled CD4+ T cells were transferred into sex- and age-matched Rag1$^{-/-}$ mice. Rag1$^{-/-}$ mice were sacrificed at day 5 after transfer, and proliferation of CD4+ T cells from the spleen was analyzed by flow cytometry.

Apoptosis assays

For the in vitro apoptosis assay, naive CD4+ T cells were activated with plate-bound anti-CD3 (0.2, 0.5, and 1.0 µg/ml) and soluble CD28 Abs (1.0 µg/ml) for 72 h. Cells were stained with Annexin V APC (BioLegend, San Diego, CA) according to the manufacturer's protocol and analyzed by flow cytometry, as previously demonstrated.[8] For the in vivo apoptosis assay, naive CD4+CD25−CD45RB^{hi} T cells were transferred into sex- and age-matched Rag1$^{-/-}$ mice. Rag1$^{-/-}$ mice were sacrificed at day 5 after transfer, and apoptosis of CD4+ T cells from the spleen was analyzed by Annexin V staining,

Multiplex ELISA

Blood was collected from recipient Rag1$^{-/-}$ mice when sacrificed, and serum was obtained and analyzed using the Mouse Cytokine/Chemokine Multiplex ELISA assay (Millipore, St. Louis, MO) according to the manufacturer's protocol and as previously described.[54]

ELISA analysis

Naive CD4$^+$ T cells (1×10^6/ml) from CK2α$^{fl/fl}$ or CK2α$^{fl/fl}$dLck-Cre mice were polarized under Th1 conditions. IFN-γ levels in the supernatant were measured using the MAX™ Standard Set Mouse IFN-γ ELISA (BioLegend, San Diego, CA).

Immunoblotting

CD4$^+$ T cells were lysed in buffer containing 1% Triton X-100 (Sigma-Aldrich), protein lysates were separated by electrophoresis, transferred to a nitrocellulose membrane, and then blotted with phospho-STAT4 (Tyr693), STAT4 (Cell Signaling Technology, Danvers, MA), and β-actin (Sigma-Aldrich, St. Louis, MO) Abs, as previously described.[9,10] Quantification of immunoblots were performed using ImageJ.

RNA isolation, RNA-seq, and quantitative RT-PCR

RNA-seq was performed as previously described.[9] Briefly, colonic CD4$^+$ T cells were sorted from recipient Rag1$^{-/-}$ mice at 6 weeks after transfer. Total RNA was extracted from FACS-sorted CD4$^+$ T cells using the miRNeasy Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocols and submitted to GENEWIZ (South Plainfield, NJ) for RNA-seq and bioinformatics analysis. RNA-seq data was submitted to the Gene Expression Omnibus Repository under accession number GSE131965. Genes with a false discovery rate <0.05 with a fold change >1.5 or <−1.5 were considered as differentially expressed genes. Further pathway analysis was performed by GSEA available through the Broad Institute.

For quantitative RT-PCR (qRT-PCR) analysis, 500–1000 ng of RNA was reverse transcribed into complementary DNA (cDNA) using M-MLV Reverse Transcriptase (Promega) as previously described.[9] cDNA was subjected to qRT-PCR using TaqMan primers (Thermo Fisher Scientific). Relative gene expression was calculated according to the ΔΔ threshold cycle (Ct) method.

Retroviral transduction

Naive CD4$^+$ T cells were activated under Th0 conditions [anti-CD3 (1 μg/ml), anti-CD28 (1 μg/ml), anti-IFN-γ (10 μg/ml) and anti-IL-4 (10 μg/ml)], and then CD4$^+$ T cells were transduced 24 h after activation by spin infection in the presence of concentrated retroviral supernatant and 1 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO). Forty-eight hours after transduction, CD4$^+$ T cells were transferred into new plates with fresh media containing 10 ng/ml IL-2 and rested for 4 days. Transduced CD4$^+$ T cells were sorted as CD4$^+$GFP$^+$ on an Aria II (BD Bioscience, San Jose, CA), 2×10^5 cells per well were stimulated with plate-bound anti-CD3 (0.1 μg/ml) and soluble anti-CD28 Abs (1 μg/ml) for 48 h, and then expression levels of Ki-67, BATF, CD40L, and ICOS were detected by FACS staining.

Statistics

Levels of significance for comparison between two groups was determined by Student's *t* test distribution and by one-sided two-sample Mann–Whitney rank-sum test for inflammation scores. Multiple comparisons were performed by ordinary one-way analysis of variance. The *p* values are indicated as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$. All error bars represent mean ± SD. All statistical analyses (excluding RNA-seq, described above) were performed using Prism software (GraphPad).

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AUTHOR CONTRIBUTIONS

W.Y., H.Q., and E.N.B. designed and analyzed the experiments. W.Y., S.A.G., Z.Y., and H.W. performed the experiments. W.Y., H.Q., and E.N.B. wrote the manuscript. J.T. and B.S. helped design and interpret experiments related to NFAT2 overexpression.

ADDITIONAL INFORMATION

The online version of this article (https://doi.org/10.1038/s41385-020-0258-x) contains supplementary material, which is available to authorized users.

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