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Graphical abstract

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The FoxO4/DKK3 axis represses IFN-γ expression by Th1 cells and limits antimicrobial immunity

Xiang Chen,1,2 Jia Hu,3,4 Yunfei Wang,5 Younghee Lee,2 Xiaohong Zhao,1 Huiping Lu,1,6 Gengzhen Zhu,1 Hui Wang,7 Yu Jiang,1 Fan Liu,1 Yongzheng Chen,1 Byung-Seok Kim,2 Qinghua Zhou,3 Xindong Liu,2 Xiaohu Wang,1 Seon Hee Chang,2 and Chen Dong1,10

1Institute for Immunology and School of Medicine, Tsinghua University, Beijing, China. 2Department of Immunology, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA. 3Lung Cancer Center, West China Hospital, Sichuan University, Chengdu, Sichuan, China. 4Department of Systems Biology, and 5Department of Melanoma Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA. 6Annonoad Gene Technology Co. Ltd., Beijing, China. 7Jiangsu Key Laboratory of Immunity and Metabolism, Department of Pathogenic Biology and Immunology, Xuzhou Medical University, Xuzhou, Jiangsu, China. 8Faculty of Dentistry, The University of Hong Kong, Hong Kong SAR, China. 9Institute of Pathology and Southwest Cancer Center, Southwest Hospital, Third Military Medical University, Chongqing, China. 10Shanghai Immune Therapy Institute, Shanghai Jiao Tong University School of Medicine–Affiliated Renji Hospital, Shanghai, China.

Forkhead box O transcriptional factors, especially FoxO1 and FoxO3a, play critical roles in physiologic and pathologic immune responses. However, the function of FoxO4, another main member of the FoxO family, in lymphoid cells is still poorly understood. Here, we showed that loss of FoxO4 in T cells augmented IFN-γ production of Th1 cells in vitro. Correspondingly, conditional deletion of FoxO4 in CD4+ T cells enhanced T cell–specific responses to Listeria monocytogenes infection in vivo. Genome-wide occupancy and transcriptomic analyses identified Dkk3 (encoding the Dickkopf-3 protein) as a direct transcriptional target of FoxO4. Consistent with the FoxO4-DKK3 relationship, recombinant DKK3 protein restored normal levels of IFN-γ production in FoxO4-deficient Th1 cells through the downregulation of lymphoid enhancer–binding factor 1 (Left) expression. Together, our data suggest a potential FoxO4/DKK3 axis in Th1 cell differentiation, providing what we believe to be an important insight and supplement for FoxO family proteins in T lymphocyte biology and revealing a promising target for the treatment of immune-related diseases.

Introduction

CD4+ helper T cells are central regulators of adaptive immune responses. After encountering a specific antigen on antigen-presenting cells, CD4+ T cells undergo clonal expansion and differentiate into functionally distinct effector subsets, including at least T helper 1 (Th1), Th2, Th17, and T follicular helper (Tfh) cells, which orchestrate immune responses against diverse microbial pathogens. Among these, IFN-γ-producing Th1 cells specialize in activating cell-mediated immune responses against intracellular pathogens and viruses. The differentiation of CD4+ T cells into Th1 cells is determined by T-bet (encoded by Tbx21), the master regulator of the Th1 differentiation program (1). Initially, T-bet is induced in response to TCR stimulation and IFN-γ/STAT1 signaling (2, 3). T-bet functions, in part, to upregulate the expression of Il12rb (encoding IL-12Rβ2), enabling developing Th1 cells to respond to IL-12 (4). As a result, a fully polarized Th1 phenotype is established by IL-12-induced STAT4 activation (5). Thus, the T-bet–STAT4 transcriptional regulatory network maintains stability of the Th1 differentiation program, ensuring CD4+ T cells receive proinflammatory signals as well as antigen stimulation to go fully committed into the Th1 cell lineage.

FoxO transcription factors belong to the family of forkhead proteins that are characterized by the presence of an approximately 100-residue forkhead DNA-binding domain. FoxO proteins function as transcriptional regulators and activate the transcription of downstream genes involved in a variety of biological processes including cellular metabolism, organ development, stress responses, and apoptosis (6, 7). In lymphoid cells, FoxO1 and FoxO3a have been shown to cooperatively regulate the generation of Foxp3+ Tregs from conventional T cells by binding to the promoter and the conserved CNS2 intronic enhancer region of the Foxp3 locus (8, 9). In addition, FoxO1 inhibits Th1 differentiation through direct binding to the Ifng gene promoter region (8). Conversely, FoxO3a drives pathogenic Th1 differentiation by inducing Eomes expression (10). In addition to Tregs and Th1 cells, FoxO transcription factors have also been reported to negatively regulate the generation of Tfh and Th17 cells. Mice with T cell–specific FoxO1 deletion accumulate a large population of Tfh cells, perhaps because FoxO1 binds to the Bcl6 gene locus and mediates its transcriptional repression (11). FoxO1 suppresses the Th17 program in vitro and in vivo by blocking RORγt binding to its target genes (12). In addition, FoxO4 has been shown to regulate insulin signaling and apoptosis (13), yet its role in lymphoid cell biology has not been well addressed.

Authorship note: XC and JH contributed equally to this work. SHC and CD contributed equally to this work and are co–senior authors.

Conflict of Interest: The authors have declared that no conflict of interest exists.

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Submitted: January 13, 2021; Accepted: July 21, 2022; Published: September 15, 2022.

Thus, our work identifies a critical axis of FoxO4/DKK3/LEF-1 in regulating Th1 cell differentiation, which is different from other FoxO family members.

Results
FoxO4 is dispensable in T cell homeostasis. To study the role of FoxO4 in T lymphocytes, we first examined its expression in naive CD4+ T cells and followed the in vitro differentiation of different T cell subsets. Although Th2, Th17, and induced Tregs (iTregs) exhibit modest levels of FoxO4 mRNA expression, FoxO4 expression was substantially higher in Th1 cells differentiated with IL-12 plus IL-2 (Figure 1A), suggesting an important role in Th1 cells.

To better investigate the function of FoxO4 in T cells, we generated T cell–specific FoxO4-deficient mice (FoxO4fl/flCd4Cre mice; referred to hereafter as FoxO4-cKO) by breeding FoxO4fl/fl mice (23) with Cd4Cre-transgenic mice, and their FoxO4fl/fl littermates (referred to as WT) were used as controls in our studies. FoxO4cKO mice, aged between 6 and 8 weeks, showed normal percentages of CD4+ single-positive (CD4 SP), CD8+ single-positive (CD8 SP), and CD4+CD8+ double-positive (CD4/8 DP) thymocytes as well as any apparent effect on T cell homeostasis.

Figure 1. FoxO4 deficiency in CD4+ T cells has no apparent effect on T cell homeostasis. (A) Real-time qPCR analysis of FoxO4 mRNA in B6 naive CD4+CD44loCD62LhiCD25 T cells differentiated for 72 hours in Th1-, Th2-, Th17-, iTreg-, and Th-f-polarizing conditions. Results are presented relative to the expression of Gapdh mRNA. (B) Flow cytometric analysis of CD4 and CD8 expression in WT and FoxO4-cKO thymocytes (n=10). The numbers adjacent to the outlined areas or in the quadrants indicate the percentage of cells. (C) Percentages of CD4 SP, CD8 SP, and CD4/8 DP cells in WT and FoxO4-cKO thymocytes (n=10). (D) Flow cytometric analysis of B220, TCRβ, CD4 (gated on TCRβ+), CD8 (gated on TCRβ+), CD44 (gated on TCRβ+CD4+ or TCRβ+CD8+), CD62L (gated on TCRβ+CD4+ or TCRβ+CD8+), and Foxp3 (gated on TCRβ+CD4+) expression on splenocytes isolated from WT and FoxO4-cKO mice (n=10). SSC, side scatter. (E) Absolute numbers of total cells, B220+ B cells, TCRβ+ T cells, CD4+ T cells, CD8+ T cells and Foxp3+ T cells in spleens from WT and FoxO4-cKO mice (n=10). Each symbol in C and E represents an individual mouse. NS, by unpaired, 2-tailed Student’s t test (C and E). Data are representative of 3 independent experiments with similar results (mean ±SD in A, C, and E).

The Dickkopf (DKK) family of glycoproteins (DKK1–4) are involved in modulating Wnt signaling pathways (14). As the best-characterized member of the DKK family, DKK1, a natural inhibitor of Wnt signaling, inhibits tumor growth and metastasis (15) and promotes Th2 differentiation (16). Moreover, DKK2 was reported to promote tumor immunity evasion through a Wnt-independent signaling pathway (17). In contrast to DKK1 and DKK2, the signaling by DKK3 is still unclear, with reports showing no effect, promotion, or inhibition of the Wnt signaling pathway (18–20). Similarly, the functional roles of DKK3 in immunity are unclear, with conflicting studies reporting its immunomodulatory or immunostimulatory functions (21, 22), suggesting that DKK3 may regulate immunity through a different mechanism.

In this study, we investigated the role of FoxO4 in T cells and found that loss of FoxO4 enhanced IFN-γ production and the effector function of Th1 cells in vitro and in vivo. Mechanistically, we identified Dkk3 as a direct transcriptional target of FoxO4, which inhibits IFN-γ production through the downregulation of lymphoid enhancer–binding factor 1 (Lef1) expression. Thus, our work identifies a critical axis of FoxO4/DKK3/LEF-1 in regulating Th1 cell differentiation, which is different from other FoxO family members.
FoxO4 negatively regulates IFN-γ production in Th1 cells in vitro.

**A** Flow cytometric analysis of naive CD4+ T cells after 3 days of polarization toward the Th0 (A), Th1 (B), Th2 (A), Th17 (A), and iTreg (A) lineages in the presence of a protein transport inhibitor in the last 5 hours \((n = 4-5)\). \(n\) is the number of experiments. Cells were gated as shown in the figure. (A) Percentage of IFN-γ-expressing WT CD4+ T cells, FoxO3-cKO CD4+ T cells, and FoxO4-cKO CD4+ T cells \((n = 5)\). Cells were gated as shown in (B). (D–H) Real-time qPCR analysis of Ifng (D), Tbx21 (E), Stat4 (F), Runx3 (G), and FoxO1 and FoxO3a (H) in CD4+ T cells isolated from WT and FoxO4-cKO mice followed by stimulation for 3 days with plate-bound anti-CD3 and anti-CD28 in Th1-polarizing conditions and assessment after restimulation with plate-bound anti-CD3 for 5 hours \((n = 3-5)\). Results are presented relative to the expression of Gapdh mRNA. *\(P < 0.05\) and ***\(P < 0.001\) by 1-way ANOVA with Tukey’s multiple-comparison test (C) or unpaired, 2-tailed Student’s t test (D–H). Data are representative of 3 independent experiments with similar results (mean ±SD in C–H).

Figure 2. FoxO4 negatively regulates IFN-γ production in Th1 cells in vitro. (A) and (B) Flow cytometric analysis of naive CD4+ T cells after 3 days of polarization toward the Th0 (A), Th1 (B), Th2 (A), Th17 (A), and iTreg (A) lineages in the presence of a protein transport inhibitor in the last 5 hours \((n = 4-5)\). (B) Flow cytometric analysis of naive CD4+ T cells after 3 days of polarization toward Th1 lineage, as described in (A) \((n = 5)\). (C) Percentage of IFN-γ-expressing WT CD4+ T cells, FoxO3-cKO CD4+ T cells, and FoxO4-cKO CD4+ T cells \((n = 5)\). Cells were gated as shown in (B). (D–H) Real-time qPCR analysis of Ifng (D), Tbx21 (E), Stat4 (F), Runx3 (G), and FoxO1 and FoxO3a (H) in CD4+ T cells isolated from WT and FoxO4-cKO mice followed by stimulation for 3 days with plate-bound anti-CD3 and anti-CD28 in Th1-polarizing conditions and assessment after restimulation with plate-bound anti-CD3 for 5 hours \((n = 3-5)\). Results are presented relative to the expression of Gapdh mRNA. *\(P < 0.05\) and ***\(P < 0.001\) by 1-way ANOVA with Tukey’s multiple-comparison test (C) or unpaired, 2-tailed Student’s t test (D–H). Data are representative of 3 independent experiments with similar results (mean ±SD in C–H).

FoxO4 negatively regulates IFN-γ production in Th1 cells in vitro.

We next assessed the role of FoxO4 in CD4+ Th cell differentiation in vitro. We sorted CD4+CD25 CD62L<sup>-</sup>CD44<sup>−</sup> naive T cells by flow cytometry and differentiated them for 3–5 days in neutral (Th0), Th1, Th2, Th17, and iTReg conditions and then analyzed the lineage-specific markers by intracellular staining (Figure 2, A and B). We observed no significant difference in Th2, Th17, or iTReg differentiation. In addition, we also examined signature genes of Th2, Th17, and iTReg in corresponding skewed conditions. Consistent with the results of intracellular staining (Figure 2A and Supplemental Figure 2, A–D), real-time quantitative PCR analysis (qPCR) (Supplemental Figure 2, E–H) revealed no significant change in signature genes at the transcriptional level for these Th cell subsets. However, FoxO4 deficiency resulted in increased IFN-γ-producing cells in the Th0 condition, in the absence of exogenous IL-12. The result indicated that FoxO4 controlled IFN-γ production inde-
mAb treatment (Supplemental Figure 3, A and B) compared with expression in WT cells. Although anti–IFN-γ treatment substantially suppressed IFN-γ expression in KO cells, it did not completely inhibit IFN-γ expression in them. This result further supports the idea that FoxO4 negatively regulates IFN-γ expression in Th1 cells.

Since the other FoxO family proteins FoxO1 and FoxO3a are also expressed and play distinct functions in Th1 cells, we suspected that FoxO4 might regulate IFN-γ through cross-regulation of FoxO family proteins. However, we did not find any difference in FoxO1 or FoxO3a expression in the absence of FoxO4 (Figure 2H), which suggests that FoxO4 probably functions in Th1 cells in a nonredundant manner. Together, FoxO4 appears to play a specific and critical role in IFN-γ production in Th1 cells in vitro.

FoxO4 deficiency enhances IFN-γ production in vivo. Having shown that FoxO4 deficiency augmented Th1 cell differentiation in vitro, we next sought to determine whether FoxO4-cKO mice independently of IL-12 signaling (Figure 2A). Since FoxO3a deficiency has been shown to repress Th1 differentiation, we next examined Th1 differentiation in FoxO4-cKO cells. Surprisingly, we found that FoxO4 deficiency, in contrast to FoxO3a deficiency, enhanced Th1 differentiation (Figure 2, B and C). To further characterize FoxO4-deficient Th1 cells, we analyzed the expression of Th1 cell signature genes by real-time qPCR, revealing dramatically augmented levels of Ifng mRNA (Figure 2D). However, the expression of Th1 lineage–specific transcription factor genes, including Tbx21, Runx3, and Stat4, in FoxO4-deficient Th1 cells was comparable to that in WT cells (Figure 2, E–G), suggesting that augmented expression of IFN-γ in FoxO4-deficient Th1 cells was not secondary to increased expression of Th1-specific transcription factors. In addition, we evaluated endogenous IFN-γ function in both WT and FoxO4-cKO cells. The intracellular staining results showed elevated IFN-γ expression in FoxO4-cKO cells with or without anti–IFN-γ mAb treatment (Supplemental Figure 3, A and B) compared with expression in WT cells. Although anti–IFN-γ treatment substantially suppressed IFN-γ expression in KO cells, it did not completely inhibit IFN-γ expression in them. This result further supports the idea that FoxO4 negatively regulates IFN-γ expression in Th1 cells.

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also produce elevated levels of IFN-γ in vivo. We immunized WT and FoxO4-cKO mice subcutaneously with keyhole limpet hemocyanin (KLH) in CFA to elicit a strong inflammatory response. One week after immunization, we isolated lymphocytes from draining lymph nodes (dLN) and found germinal center (GC) B cells (B220+GL7+CD95+) and Tfh cells (CD4+CD44hiCXCR5+PD-1+) developed normally in FoxO4-cKO mice (Supplemental Figure 4, A and B), indicating that FoxO4 was dispensable for the development of Tfh cells and GC reactions. In addition, the production levels of KLH-specific antibody isotypes including IgA, IgM, IgG1, and IgG2a were similar between WT and FoxO4-cKO mice, (Supplemental Figure 4C). Furthermore, cell proliferation (Supplemental Figure 4D) as well as IL-2 production (Supplemental Figure 4E) in response to rechallenge with KLH were similar in CD4+ T cells from WT and cKO mice, indicating that FoxO4 was dispensable in regulating T cell priming and proliferation in vivo. However, intra- cellular cytokine staining showed that, in response to KLH restimulation, FoxO4-deficient CD4+ T cells in dLN produced higher levels of IFN-γ (Figure 3A) with substantially increased frequencies of IFN-γ–producing CD4+ T cells (Figure 3B) compared with WT cells. Consistent with these results, ELISAs revealed that after restimulation with KLH, T cells from FoxO4-deficient mice had significantly higher expression of IFN-γ, but similar expression levels of IL-17A compared with T cells from immunized WT mice (Figure 3C),
which suggested that FoxO4 deficiency in T cells selectively altered IFN-γ production in vivo. Together, FoxO4 appeared to negatively regulate CD4+ T cells in their IFN-γ production in antigen-specific T cell responses in vivo.

Loss of FoxO4 enhances Th1 cell-mediated immunity to bacterial infection. To further investigate the role of FoxO4 in T cell-mediated antipathogen immune responses in vivo, we next applied an infection model using an intracellular bacterial pathogen, Listeria monocytogenes, which is known to induce strong T cell responses by the induction of IFN-γ–producing Th1 cells and CD8+ effector T cells. We infected age- and sex-matched WT and FoxO4-cKO mice using a modified Lm-OVA strain (L. monocytogenes strain expressing OVA). Seven days after infection, as expected, FoxO4-cKO mice showed significantly reduced bacterial burdens in the liver and spleen compared with WT mice (Figure 3D), indicating that the FoxO4-cKO mice were more resistant to L. monocytogenes infection.

To examine pathogen-specific CD4+ and CD8+ effector T cell responses during infection, we restimulated splenocytes and liver cells from infected mice with an MHC class II–restricted listeriolysin O peptide (LLO 190–201) and an MHC class I–restricted OVA peptide (OVA257–264 (SIINFEKL)), respectively. As expected, Lm-OVA–infected FoxO4-cKO mice, compared with WT mice, had substantially more CD4+ and CD8+ IFN-γ–producing effector T cells following restimulation with LLO 190–201 (Figure 3, E and F) and OVA 257–264 (Figure 3, E and G), respectively. To determine whether the phenotypic difference in Listeria infection between WT and cKO mice was dependent on CD4+ T cells, we performed antibody-mediated depletion of CD4+ T cells during Listeria infection. Bacterial burden enumeration showed no difference in CFU in the livers or spleens of CD4-depleted WT and FoxO4-cKO mice, but both had higher CFU compared to normal WT and FoxO4-cKO mice, respectively (Supplemental Figure 5). Taken together, our findings indicate that FoxO4 serves as a negative regulator of antigen-specific Th1 cell responses to bacterial infection. Thus, loss of FoxO4 enhanced Th1 cell–mediated immunity to bacterial infection in vivo.

Ectopic FoxO4 expression represses IFN-γ production in Th1 cells. To assess the function of FoxO4 in processes of Th1 differentiation, we performed an in silico search for FoxO transcription factor–binding sites in the FoxO4 locus (Supplemental Figure 6). Together, these data suggested that FoxO4 is a direct transcriptional target of FoxO4.

Next, we performed ChIP-Seq assay and identified 515 FoxO4–binding peaks using the model-based analysis for ChIP-Seq (MACS) algorithm. The results showed extensive binding of FoxO4 at exon and transcription start site (TSS) upstream regions (Figure 4E). Next, we performed an in silico search for FoxO4 transcription factor–binding sites using binding profiles from the JASPAR CORE database (jaspar.genereg.net). We found that 26.6% (137 of 515) of the FoxO4 peaks contained a FoxO-binding motif and were considered to be high-confidence binding sites of FoxO4 (Figure 4F). Intersecting with the microarray data above, 17 genes containing high-confidence FoxO4–binding peaks were upregulated in RV-FoxO4–infected Th1 cells: Arvfc, Cnn3, Dkk3, Ab24611, Tmem88b, Gng7, Mami13, Ezhl, Mgt5b, Rsph4a, Dat, Abegl, Spib, Tsk4, Trdn, Mypl, and Hrh1. In addition, 9 genes containing high-confidence FoxO4–binding peaks were downregulated by ectopic expression of FoxO4: Styk1, Cth, Fastk1, Fosl2, Zfp286, Gnal, Ankrd27, Inntm, and Mboat4 (Figure 4G). Among these potential FoxO4 target genes, Dkk3, one member of the Dickkopf family, drew our attention, since DKK family proteins have been shown to regulate CD4+ T cell–mediated immune responses (24, 25). More important, the predicted binding motif for FoxO proteins (JASPAR MA0848.1) with a score of 11.3 (Figure 4H) and specific FoxO4–binding peaks were found at the Dkk3 locus (Figure 4I). Together, these data suggested that Dkk3 is a potential direct target of FoxO4 protein.

Dkk3 restores normal IFN-γ production in FoxO4-deficient Th1 cells through downregulating Lef1 expression. Having identified Dkk3 as potential target of FoxO4, we first examined whether Dkk3 expression in CD4+ T cell is regulated by FoxO4. While we found no significant difference in Dkk3 expression levels between WT and FoxO4-cKO Th1 cells 48 hours after differentiation, in a time-course analysis, Dkk3 expression levels were indeed lower in FoxO4-deficient cells between 0 and 8 hours under Th1 cell differentiation conditions (Figure 5A). To further confirm that FoxO4 regulates Dkk3 at the early stage of Th1 differentiation, we performed Western blotting to analyze DKK3 protein expression at several time points after activation under Th1-polarizing conditions (Figure 5B). Together with the qPCR data (Figure
5A), we could make a conclusion that, from 0–8 hours after activation, compared with WT cells, both mRNA and protein expression levels of Dkk3 were decreased in FoxO4-KO cells, suggesting that FoxO4 played a critical role in regulating Dkk3 transcription at the early stage of Th1 differentiation.

Next, we investigated whether treatment with DKK3 protein could overcome the inhibitory effect of FoxO4 on IFN-γ production in Th1 cells. We assessed the dose-dependent response of DKK3 on IFN-γ expression and compared it with Wnt-C59, a known Wnt/β-catenin inhibitor. Intracellular cytokine staining showed that DKK3 (50247-M08H, Sino Biological) at concentrations greater than 30 ng/mL could inhibit IFN-γ expression, similar to what we observed with Wnt-C59 (S7037, Selleck), in WT cells; however, in FoxO4-cKO cells, 15 ng/mL DKK3 was sufficient to override the effect of FoxO4 deficiency on IFN-γ expression (Figure 5, C and D), indicating that DKK3 treatment could restore normal IFN-γ production in FoxO4-deficient Th1 cells. Next, in order to validate the function of the FoxO4/DKK3 axis in vivo, we treated WT and FoxO4-cKO mice with DKK3 and the Wnt inhibitor Wnt-C59, respectively, in the Listeria infection model. Data showed that DKK3, but not the Wnt inhibitor, significantly increased bacteria burdens in the livers and spleens of FoxO4-cKO mice (Supplemental Figure 8), suggesting a feedback regulation of DKK3 in FoxO4-deficient mice and an unclear mechanism whereby DKK3 functions in a Wnt-independent manner. Together, our current findings demonstrate that the FoxO4/DKK3 axis plays a critical role in regulating T cell immunity for L. monocytogenes infection.
The Journal of Clinical Investigation

RESEARCH ARTICLE

J Clin Invest. 2022;132(18):e147566  https://doi.org/10.1172/JCI147566

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Data published by other groups showed that β-catenin overexpression elevates the production of IFN-γ, and DKK1 promotes pathological Th2 cell-mediated inflammation (16). Therefore, we hypothesized that DKK3 could attenuate IFN-γ production by inhibiting the Wnt signaling pathway. Surprisingly, real-time qPCR results showed that, in WT cells, DKK3 treatment failed to downregulate the expression of genes relevant to the Wnt signaling pathway as broadly as Wnt-C59 did (Figure 6A). In FoxO4-cKO cells, we found that DKK3 treatment could attenuate the expression of Lef1, which encodes the transcriptional factor LEF-1, among relevant genes in the Wnt signaling pathway (Figure 6A). Although we did not find a difference in Lef1 expression 24 hours after Th1 differentiation, it is still possible that there was a specific time period in which Lef1 expression was different between WT and FoxO4-deficient Th1 cells. Thus, we assessed Lef1 mRNA expression levels at several time points at the early stage of Th1 differentiation in WT and FoxO4-cKO cells (Figure 6B). We found that in the first 2 hours after T cell activation, compared with WT cells, Lef1 expression in FoxO4-cKO cells was significantly increased, and then dropped to baseline levels in both cell types, suggesting that Lef1 expression was regulated by FoxO4 at the early stage of Th1 differentiation.

Previous studies showed that Tcf7, which encodes the transcriptional factor T cell factor 1 (TCF-1), inhibits Th1 cell differentiation and the production of IFN-γ in a Wnt/β-catenin-independent manner (26). Moreover, Lef1, as another main downstream gene of the Wnt/β-catenin signaling pathway, has been shown to play a role opposite that of Tcf7 in T cell development and malignancy (27). Therefore, it is possible that the FoxO4/DKK3 axis represses IFN-γ production by Th1 cells by regulating Lef1 expression. To test our hypothesis, we conducted retroviral transduction with a vector encoding Lef1 in FoxO4-cKO cells treated with DKK3.

Figure 6. The DKK3/LEF-1 axis negatively regulates IFN-γ expression in Th1 cells. (A) Real-time qPCR analysis of the expression of Ifng, Tbx21, Tcf7, Lef1, Tcf4, and Myc in naive CD4+ T cells after 2.5 days of polarization toward the Th1 lineage in the presence of Wnt-C59 (10 nM or 100 nM), a Wnt/β-catenin inhibitor, and DKK3 protein (15–60 ng/mL) (n = 3). (B) Real-time qPCR analysis of Lef1 expression in cultured CD4+ T cells from WT and FoxO4-cKO mice in the Th1-polarizing condition at different time points (n = 3). (C) Intracellular staining for IFN-γ in naive CD4+ T cells transduced with an empty vector (RV) or a vector encoding LEF-1 (RV-LEF-1) after 2.5 days of polarization toward the Th1 lineage, with or without 30 ng/mL DKK3 protein treatment (n = 5). (D) Frequency of IFN-γ–expressing cells as in C (n = 5). **P < 0.01 and ***P < 0.001, by 2-way ANOVA with Tukey’s multiple-comparison test (A and D) or unpaired, 2-tailed Student’s t test (B). Data are representative of 2 (A and B) or 3 (C and D) independent experiments (mean ±SD in A, B, and D).
Intracellular cytokine staining showed that DKK3 treatment impaired IFN-γ expression in FoxO4-cKO cells transduced with the empty vector (Figure 6, C and D). In contrast, infection with the vector encoding Lefty greatly augmented IFN-γ expression in DKK3-treated FoxO4-cKO cells compared with FoxO4-cKO cells under Th1-polarizing conditions (Figure 6, C and D), indicating that ectopic Lefty expression in FoxO4-cKO cells could rescue IFN-γ expression after DKK3 treatment. In summary, FoxO4 deficiency enhanced IFN-γ expression via downregulation of Dkk3 and subsequent elevation of Lefty expression. Thus, our data demonstrate a potential FoxO4/DKK3/LEF-1 axis in the regulation of IFN-γ production by Th1 cells.

Discussion

FoxO transcription factors regulate basic cellular processes, such as energy metabolism, stress responses, and apoptosis. In the immune system, FoxO proteins control critical molecules of T cell homeostasis and tolerance (28). Our study reveals a regulatory role of FoxO4 in Th1 cells that is different from that of FoxO1 and FoxO3a and identifies Dkk3 as a direct transcriptional target of FoxO4 in Th1 cells. In Th1 cells, DKK3, a putative Wnt antagonist, suppressed IFN-γ production by regulating Lefty expression. Thus, we identified a potential FoxO4/DKK3/LEF-1 axis in the regulation of IFN-γ expression during Th1 cell differentiation, providing what we believe to be an important insight and supplement for FoxO family proteins in T lymphocyte biology. Given that our study indicates a potential regulatory axis of FoxO4/IFN-γ in Th1 cells, all 3 of the FoxO family members are likely mediators of nonredundant and specific functions in T cell biology.

To understand how FoxO4 suppresses IFN-γ expression, we explored genome-wide occupancy of FoxO4 in Th1 cells. Although we identified more than 20 molecules targeted by FoxO4, which suggested the presence of additional targets of FoxO4, we pursued an in-depth investigation of the FoxO4/DKK3 axis because of previous studies linking DKKs and CD4+ T cell responses (29). In our study, our data suggest that CD4+ T cells are another potential source for functional DKK3. However, to demonstrate whether DKK3 derived from CD4+ T cells plays a suppressive role in Th1 cell functions in a nonredundant manner, studies using genetic strains with specific deletion of Dkk3 in T cells may be required.

Although DKK1 and DKK2 function as Wnt antagonists, the signaling by DKK3 is still unclear, with reports showing no effect, promotion, or inhibition of the Wnt signaling pathway (18–20). Similarly, the functional roles of DKK3 in immunity are unclear, with conflicting studies reporting its immunomodulatory or immunostimulatory functions (21, 22), suggesting DKK3 may regulate immunity through a different mechanism. In our study, we found Wnt-C59, a potent Wnt inhibitor, suppressed diverse sets of genes linked to Wnt signaling in Th1 cells; however, DKK3 did not inhibit Wnt-related molecules as broadly as did the Wnt antagonist. This finding raised the possibility that DKK3 regulates IFN-γ production in Th1 cells through a Wnt-independent pathway. Although DKK3 did not inhibit Wnt-related molecules as broadly as the Wnt antagonist did, we showed that Lefty expression was suppressed by DKK3 in Th1 cells. Both TCF-1 and LEF-1 are downstream effectors of the Wnt signaling pathway and are essential for early T cell development (26, 27). Compared with TCF-1, LEF-1 is expressed predominantly in Th1 cells and was originally identified as a lymphoid-specific DNA-binding protein that recognizes a 5′-CCTTTGAA motif in the TCRα enhancer (31). Previous studies showed that TCF-1 and LEF-1 have cooperative and opposing roles with increased IFN-γ production (29). However, the direct target cells of DKK3 were not reported in this study. Surprisingly, another study has shown an opposite function of DKK3, in which DKK3 promoted Th1 cell differentiation and increased IFN-γ production indirectly via DCs (30). In our study, we found an inhibitory function of DKK3 for IFN-γ production in CD4+ T cells and identified FoxO4 as a regulator of DKK3. Furthermore, our data extend the function of DKK3 in T cells to that of a regulator of IFN-γ and established DKK3 as a T cell–derived molecule that can be induced by FoxO4 activation in Th1 cells. It is currently not clear whether the FoxO4/DKK3 axis in T cells is required during autoimmunity or other infectious diseases, which warrants further investigation. In addition, we found that mRNA expression of Dkk3 was only modestly perturbed in the FoxO4-cKO mice, although the protein levels were more significantly altered. We currently do not know why there was a difference in mRNA versus protein levels at the indicated time points. However, we can speculate that (a) the stability of DKK3 mRNA and protein expression could be influenced by additional epigenetic and translational regulation, and that (b) FoxO4 probably functions at the early stage of Th1 cell differentiation, which is supported by our Western blot data showing that Foxo4-deficient cells produced less DKK3 in a short period after stimulation. Thus, substantial mRNA expression differences, if they exist, between WT and cKO cells may be detectable in a specific, narrow time window. These possibilities may lead to more significant differences in protein, but not mRNA, expression levels in cKO cells.

Previous studies showed different sources of DKK3, for example, CD8+ T cells secreting DKK3 was identified as an essential molecule for T cell tolerance (21). In addition, tissue-derived DKK3 functions as a modulator of local CD4+ and CD8+ T cell responses (29). In this study, our data suggest that CD4+ T cells are another potential source for functional DKK3. However, FoxO4 may be detectable in a specific, narrow time window. These possibilities may lead to more significant differences in protein, but not mRNA expression levels in cKO cells.
in T cell development (27), and in most cases, TCF-1 appeared to have a dominant effect (32). However, here we demonstrated a TCF1-independent function for LEF-1 in FoxO4-deficient Th1 cell differentiation. In our study, reconstitution of LEF-1 in FoxO4-deficient T cells restored DKK3-mediated IFN-γ suppression, linking the regulatory circuit of FoxO4/DKK3/LEF-1/IFN-γ.

In addition, there are several limitations in our current study. The mechanisms of DKK3 regulation of LEF-1 expression and LEF-1 promotion of IFN-γ expression remain unclear. Identification of DKK3 receptors on the cell surface may help to address these questions, which merit further study.

In conclusion, this study identified a critical role of FoxO4 in the regulation of IFN-γ production in Th1 cells. The regulatory axis of FoxO4/DKK3/LEF-1/IFN-γ was critical for host defense during acute infection. Our findings provided a basis for further investigation into how FoxO family proteins control the differentiation and function of CD4+ T cells and other lymphocyte lineages. Additionally, our results identified a potential target for therapeutic manipulation of acute infection and autoimmune disease.

Methods

Mice. Mice with floxed FoxO4 alleles (FoxO4fl/fl) and FoxO3 alleles (FoxO3fl/fl) were previously generated (23) and were provided as gifts by Ronald A. DePinho (MD Anderson Cancer Center, Houston, Tex., USA). C57BL/6 and Cd4cre-transgenic mice (B6 background) were provided as gifts by The Jackson Laboratory and the National Cancer Institute (NCI), NIH, respectively. Mice with T cell-specific deletion of FoxO4 were generated by crossing FoxO4fl/fl mice with Cd4cre-transgenic mice. The FoxO4fl/fl Cd4cre (FoxO4-cKO) mice and FoxO3fl/fl (WT) littermates on the mixed background were used in the experiments. The FoxO4fl/fl Cd4cre (FoxO3-cKO) mice were generated in a similar manner. All mice were maintained in specific pathogen-free animal facilities at MD Anderson Cancer Center and Tsinghua University, and all animal experiments were carried out using 6- to 10-week-old mice.

Naïve T cell stimulation and differentiation in vitro. Naïve CD4+CD25 CD62LhiCD44lo T cells from spleens and lymph nodes were isolated using a FACS Aria sorter (BD Biosciences). Purified naïve T cells were stimulated with 2 μg/mL plate-bound anti-CD3 (2C11, Bio X Cell) and 2 μg/mL anti-CD28 (37.51, Bio X Cell) in the presence of 5 μg/mL anti-IFN-γ (XMG1.2, Bio X Cell), 5 μg/mL anti-IL-4 (11B11, Bio X Cell), and 40 U/mL IL-2 (Peprotech) for the generation of Th0 cells; 20 ng/mL IL-12 (Peprotech), 5 μg/mL anti-IL-4, and 40 U/mL IL-2 for the generation of Th1 cells; 10 ng/mL IL-4 (Peprotech), 10 μg/mL anti-IFN-γ and 40 U/mL IL-2 for the generation of Th2 cells; 1 ng/mL TGF-β1 (Peprotech), 10 ng/mL IL-6 (Peprotech), 5 μg/mL anti-IFN-γ, and 5 μg/mL anti-IL-4 or 10 ng/mL IL-23, 10 ng/mL IL-1β (Peprotech), 10 ng/mL IL-6, 5 μg/mL anti-IFN-γ, and 5 μg/mL anti-IL-4 for the generation of Th17 cells; and 1 ng/mL TGF-β1, 5 μg/mL anti-IFN-γ, 5 μg/mL anti-IL-4, and 40 U/mL IL-2 for the generation of iTregs. Cells were cultured in complete medium (RPMI medium containing 10% FBS, supplemented with penicillin-streptomycin, HEPES, L-glutamine, sodium pyruvate, and 2-mercaptoethanol) for 3-5 days, followed by intracellular staining and RNA preparation.

Intracellular staining and flow cytometry. Cells were restimulated for 5 hours with PMA (50 ng/mL; MilliporeSigma), ionomycin (500 ng/mL; MilliporeSigma), and monensin (Golgistop, BD Biosciences). After staining for cell-surface markers, intracellular staining was performed according to the manufacturer’s protocols (Cytotox/Cytoperm buffer set from BD Biosciences; Foxp3 staining buffer set from eBioscience). The flow cytometer FACSCalibur or LSRFortessa (both from BD Biosciences) and FlowJo software (Tree Star) were used for flow cytometry and data analysis. All antibodies used in this study are listed in Supplemental Table 1.

Retroviral transduction. FoxO4 and LEF-1 cDNA were PCR amplified and subcloned into an RVKM retroviral vector (33). Retrovirus production was carried out in Plat-E cells (ATCC) as described previously. Naïve CD4+CD25 CD62LhiCD44hi T cells were isolated and stimulated with plate-bound anti-CD3 and anti-CD28 antibodies. Twenty hours after stimulation, the supernatant with virus particles was added, and spin transduction was performed in the presence of polybrene (8 μg/mL) at 900 x g for 90 minutes at 30°C. Next, intracellular staining, ELISA, and real-time qPCR were carried out after 4-5 days of culturing in the appropriate Th cell-polarizing conditions. For some applications, GFP+ and GFP+ cells were sorted with a BD FACS Aria.

ELISA and real-time qPCR. Cytokines were measured by ELISA as we described previously (33). Total RNA was extracted using TRIzol reagent (Invitrogen, Thermo Fisher Scientific). For analysis of mRNA transcripts, cDNA was generated by oligo(dT) priming and Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen, Thermo Fisher Scientific) and amplified in IQ SYBR Green Supermix (Bio-Rad) in the presence of specific primer pairs. Data were normalized to the GAPDH gene for each sample. All primer sequences used in this study are listed in Supplemental Table 2.

KLH immunization. Mice were immunized subcutaneously with 1 mg/mL KLH emulsified in 0.5 mg/mL CFA (100 μL per mouse). After 1 week of immunization, mice were sacrificed, and a series of analyses were performed. Briefly, KLH-specific IgA, IgM, IgG1, and IgG2a levels in serum were measured by ELISA using the SBA Clonotyping System (Southern Biotech). For intracellular cytokine staining, lymphocytes from splenocytes and splenocytes were stimulated with 0 and 100 μg/mL KLH for 24 hours in the presence of monensin in the last 5 hours, and then IFN-γ and IL-17A-producing cells were detected in the CD4+ cell population as described above. For analysis of cytokine expression, lymphocytes in splenocytes were stimulated with 0, 5, 20, and 100 μg/mL KLH for 72 hours, followed by ELISA detection of IFN-γ and IL-17A in the supernatant.

L. monocytogenes infection. An erythromycin-resistant strain of Lm-ova (recombinant L. monocytogenes expressing a truncated OVA protein, aa 134–387) was grown in brain heart infusion media supplemented with 5 μg/mL erythromycin. Age- and sex-matched WT and FoxO4-cKO mice were intravenously injected with 105 CFU Lm-OVA in 100 μL PBS and sacrificed on day 7 after injection. The number of live bacteria lysates from infected organs was determined by measuring CFU. In brief, the livers and spleens were collected and homogenized, the homogenates were spread on brain heart infusion agar, and L. monocytogenes CFU were assessed after overnight growth at 37°C. For analysis of specific T cell responses, splenocytes and liver cells were stimulated with OVA257–264 (SIINFEKL) or LLO190–201 (NEKYQA-YPNVS) peptide overnight, in the presence of monensin in the last 5 hours, for intracellular cytokine staining or for 3 days for ELISA. For CD4+ T cell depletion, infected mice were treated with anti-CD4 antibody (GK1.5, Bio X Cell) intraperitoneally on days -1 and +1 respectively.
to the challenge with Lm-OVA. Livers and spleens were harvested on day 4 after infection for enumeration of bacterial burden. For chemical treatment, prior to WT and FoxO4-cKO mice were infected with Lm-OVA on day 0, mice were treated with Wnt-C59 (10 mg/kg) intravenously and DKK3 (50 μg/kg) intraperitoneally, respectively, for 3 consecutive days, from day -3 to day -1. Then the bacterial burdens in spleens and livers from each group were compared on day 8.

Microarray. Naïve T cells were activated and infected with RV and RV-FoxO4, respectively. Total RNA was extracted from sorted GFP+ cells, and Agilent 028005 SurePrint G3 Mouse GE 8×60K Microarrays were used to probe global gene expression changes.

ChiP and ChiP-Seq. The ChiP experiment was performed using Active Motif’s ChiP assay kit (catalog 53035) as we described previously (33), with anti-FoxO4 antibody (sc-5221, Santa Cruz Biotechnology) or rabbit IgG (ab37415, Abcam). The precipitated DNA was quantified by real-time qPCR with a Bio-Rad CFX96 real-time system using the primer specific for different targets. Primers for FoxO4 ChiP-qPCR were synthesized as reported previously (10).

For ChiP-Seq, input and endogenous FoxO4 ChiPed DNA obtained by the ChiP procedure above were subjected to library preparation using the NEXTflex ChiP-Seq DNA Sequencing Kit (5143, Bioo Scientific). The DNA libraries were sequenced on an Illumina HiSeq 2500 by Bionova Biotech Co. Ltd. For data analysis, the raw reads were mapped to the mm10 genome using bowtie (34), and then MACS software was used for peak calling. The motifs were called from significantly enriched peaks using the MEME suite (35). An in-house script was used to calculate the normalized and input-subtracted depth. The files were visualized in the UCSC Genome Browser. The microarray and ChiP-Seq data sets were deposited in the NCBI’s Gene Expression Omnibus (GEO) database (GEO GSE133035).

Statistics. All statistical analyses were performed with an unpaired, 2-tailed Student’s t test or 1-way or 2-way ANOVA where appropriate using GraphPad Prism 8 (GraphPad Software). A P value of less than 0.05 was considered statistically significant. Data are presented as the mean ± SD.