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ETV5 promotes lupus pathogenesis and follicular helper T cell differentiation by inducing osteopontin expression

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Follicular helper T (T_{FH}) cells mediate germinal center reactions to generate high affinity antibodies against specific pathogens, and their excessive production is associated with the pathogenesis of systemic autoimmune diseases such as systemic lupus erythematosus (SLE). ETV5, a member of the ETS transcription factor family, promotes T_{FH} cell differentiation in mice. In this study, we examined the role of ETV5 in the pathogenesis of lupus in mice and humans. T cell–specific deletion of *Etv5* alleles ameliorated T_{FH} cell differentiation and autoimmune phenotypes in lupus mouse models. Further, we identified *SPP1* as an ETV5 target that promotes T_{FH} cell differentiation in both mice and humans. Notably, extracellular osteopontin (OPN) encoded by *SPP1* enhances T_{FH} cell differentiation by activating the CD44-AKT signaling pathway. Furthermore, *ETV5* and *SPP1* levels were increased in $CD4^+$ T cells from patients with SLE and were positively correlated with disease activity. Taken together, our findings demonstrate that ETV5 is a lupus-promoting transcription factor, and secreted OPN promotes T_{FH} cell differentiation.

follicular helper T cell | lupus | ETV5 | *SPP1* | osteopontin

Significance

Systemic lupus erythematosus (SLE) is an antibody-mediated autoimmune disease. The excessive production of follicular helper T (T_{FH}) cells, which play a vital role in helping B cells produce antibodies, is a feature of SLE. While much is known about T_{FH} development, a deeper understanding of the molecular mechanisms controlling T_{FH} cell differentiation and functions will help in developing effective treatments for SLE. Our study identifies the ETV5 transcription factor as a key regulator of T_{FH} cell differentiation in both mice and humans. Additionally, we emphasize the role of secreted osteopontin (OPN), controlled by the ETV5-regulated *SPP1* gene, in promoting T_{FH} cell differentiation through the CD44-AKT signaling pathway. These findings highlight ETV5 and OPN as potential therapeutic targets for SLE.

Systemic lupus erythematosus (SLE) is one of the most common antibody-mediated autoimmune diseases, in which multiple organs are targeted and damaged by the abnormal functioning of the immune system (1). This results in a wide array of symptoms in individual patients, including skin rashes, splenomegaly, and glomerulonephritis (1). The hallmark of SLE is the aberrant production of autoantibodies. Consequently, therapeutic strategies have been developed primarily targeting B cells that produce antibodies (2). Additionally, other immune cells that facilitate B cell proliferation and differentiation, such as macrophages, dendritic cells (DCs), and T cells, have garnered attention (3–6). SLE is a highly heterogeneous autoimmune disease (7), necessitating a personalized approach to the treatment and care of each patient. Therefore, a detailed analysis of the roles and subsets of immune cells involved and an in-depth investigation of the molecular mechanisms underlying SLE pathogenesis are important for tailoring personalized treatment strategies.

Follicular helper T (T_{FH}) cells are a specialized subset of $CD4^+$ T helper (T_H) cells that are pivotal in orchestrating adaptive immune responses. T_{FH} cells reside primarily in the germinal centers (GCs) of secondary lymphoid organs and mediate GC responses that promote the affinity maturation of B cells and plasma and memory B cell differentiation (8–10). T_{FH} cell differentiation is a tightly regulated process that is influenced by a multitude of factors. Cytokines, most notably interleukin (IL)-6 and IL-21 (mice) or IL-12 (humans), are key drivers in T_{FH} cell differentiation (8–10). These cytokines promote the expression of BCL6 (11, 12), essential for T_{FH} cell development, maintenance, and function (13–16). Concurrently, Blimp-1, an antagonist of BCL6, counterbalances T_{FH} cell differentiation, ensuring the controlled progression of this process (15, 16). Additionally, the inducible T cell costimulator (ICOS) signaling pathway plays an essential role in the differentiation and function of T_{FH} cells (17). The interaction between ICOS and its ligand ICOSL on B cells activates the PI3K-AKT pathway in T_{FH} cells, thereby regulating the expression of genes required for the localization of T_{FH} cells within B cell follicles, as well as BCL6 (16–18). Once localized within the GCs, T_{FH} cells play a pivotal role by providing crucial signals to B cells. These signals include the CD40-CD40L interaction and secretion of IL-21, which collectively contribute to B cell maturation and affinity maturation of antibodies, enabling the production of high affinity antibodies targeting specific pathogens (8, 9). Given that T_{FH} cells serve as central players in antibody production by B cells, their close association with the pathogenesis of antibody-mediated autoimmune diseases, such as SLE, is evident (19–21). Aberrations in the development and function of T_{FH} cells can lead to

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the excessive production of autoantibodies by B cells, ultimately resulting in systemic damage to multiple organs (22–24). Therefore, identifying and modulating the mechanisms that govern the differentiation and function of T_{FH} cells in autoimmune contexts holds promise as a potential treatment for autoimmune diseases.

ETS translocation variant 5 (ETV5) is a member of the PEA3 group transcription factor family, which is part of the ETS transcription factor superfamily (25). The *PEA3* group genes, including *ETV1*, *ETV4*, and *ETV5*, are known as targets of the transcriptional repressor capicua (CIC) in mammals (26–28). Like other ETS transcription factors, ETV5 regulates the expression of target genes by binding to the GGAA/T motif via its ETS domain (29). Multiple studies have provided compelling evidence for the involvement of ETV5 in regulating $CD4^+$ and $CD8^+$ T cell immunity. ETV5 promotes the differentiation of liver-resident memory-like $CD8^+$ T cells and liver injury in mice by inducing the expression of *Hobit*, a key transcription factor in tissue-resident memory T cell development (30). ETV5 controls T_H17 cell differentiation by directly promoting *Il17a* and *Il17f* expression, leading to T cell–dependent allergic airway inflammation in mice (31). ETV5 also regulates IL-10 and IL-9 in T_H2 and T_H9 cells, respectively (32, 33). Furthermore, our previous study showed that CIC deficiency–mediated ETV5 derepression promotes T_{FH} cell differentiation in mice (34). *Cic*-deficient mice display lupus-like autoimmune phenotypes (34). Nevertheless, it remains unclear whether these phenotypes are direct consequences of ETV5 derepression. Furthermore, whether ETV5 regulates T_{FH} cell differentiation and lupus pathogenesis in humans has not been investigated.

In this study, we explored the role of ETV5 in lupus pathogenesis using multiple lupus mouse models and samples from human SLE patients. Our study demonstrated that ETV5 is a transcription factor that promotes T_{FH} cell differentiation and lupus pathogenesis in both mice and humans. Additionally, we identified *SPP1*, encoding osteopontin (OPN), as an ETV5 target gene that promotes T_{FH} cell differentiation. Intriguingly, we found that extracellular OPN promotes T_{FH} cell differentiation by activating the CD44-AKT pathway, revealing a unique molecular mechanism by which OPN controls T_{FH} cell differentiation.

Results

Recovery of Autoimmune-Like Phenotypes in T Cell–Specific *Cic* and *Etv5* Double-Null Mice. Our previous study showed that T cell–specific *Cic*-null (*Cic$^{f/f}$;Cd4-Cre*) mice develop systemic autoimmunity accompanied by the expansion of T_{FH} and GC B cell populations (34). We also demonstrated that *Etv5* is a CIC target gene that promotes T_{FH} cell differentiation (34). To comprehensively understand the role of ETV5 in T cells, we generated T cell–specific *Etv5*-null (*Etv5$^{f/f}$;Cd4-Cre*) mice and analyzed their T cell subsets, including T_{FH} cells. As expected, the frequency of T_{FH} cells significantly decreased in the spleens of *Etv5$^{f/f}$;Cd4-Cre* mice, regardless of immunization with ovalbumin (OVA) in alum (*SI Appendix*, Figs. S1 *A* and *B* and S2 *A*–*C*). Consistent with these results, the frequency of GC B cells also significantly decreased (*SI Appendix*, Figs. S1*C* and S2*D*). Among the T_{FH} subsets, the frequency of IFN-γ^+ $T_{FH}1$ cells significantly decreased in *Etv5$^{f/f}$;Cd4-Cre* mice (*SI Appendix*, Fig. S1*D*). Consistent with previous findings (31), the frequency of T_H17 cells significantly decreased in the spleens of *Etv5$^{f/f}$;Cd4-Cre* mice (*SI Appendix*, Fig. S1*E*), whereas the proportions of T_H1, T_H2, and regulatory T (T_{REG}) cells were comparable between *Etv5$^{f/f}$* (WT) and *Etv5$^{f/f}$;Cd4-Cre* mice (*SI Appendix*, Fig. S1 *E* and *F*).

To examine whether ETV5 derepression is responsible for CIC deficiency–induced autoimmune-like phenotypes and the expansion of the T_{FH} cell population, we generated T cell–specific *Cic* and *Etv5* double-null (*Cic$^{f/f}$;Etv5$^{f/f}$;Cd4-Cre*) mice. Consistent with our previous results (34), the increased frequency of T_{FH} and GC B cells in *Cic$^{f/f}$;Cd4-Cre* mice was significantly suppressed in *Cic$^{f/f}$;Etv5$^{f/f}$;Cd4-Cre* mice (Fig. 1 *A*–*C*). The $CD11c^+$T-bet^+ age-associated B cell population, which is known to expand in lupus mouse models and patients with SLE (35), was dramatically increased in *Cic$^{f/f}$;Cd4-Cre* mice, while this phenomenon was significantly attenuated in *Cic$^{f/f}$;Etv5$^{f/f}$;Cd4-Cre* mice (Fig. 1*D*). Autoimmune-like phenotypes, including increased serum immunoglobulin (Ig) and anti-dsDNA antibody levels, tissue infiltration of immune cells, and IgG and C3 deposition in the kidney glomeruli, substantially recovered in *Cic$^{f/f}$;Etv5$^{f/f}$;Cd4-Cre* mice (Fig. 1 *E*–*H*). These data demonstrate that CIC deficiency–induced ETV5 derepression promotes T_{FH} cell differentiation and systemic autoimmunity.

ETV5 Deficiency Suppresses T_{FH} Cell Differentiation and Autoimmunity in Lupus Mouse Models. Next, we extended our findings to investigate whether ETV5 promotes lupus pathogenesis. To this end, we induced lupus-like autoimmunity in WT and *Etv5$^{f/f}$;Cd4-Cre* mice by intraperitoneal injection of tetramethylpentadecane (commonly known as pristane) (36) and analyzed them 4 mo after injection (Fig. 2*A*). Pristane treatment–induced lupus phenotypes, including splenomegaly, increased serum Ig and anti-dsDNA antibody levels, tissue infiltration of immune cells, glomerular necrosis, and IgG and C3 deposition in the kidney glomeruli, were substantially alleviated in *Etv5$^{f/f}$;Cd4-Cre* mice compared to WT mice (Fig. 2 *B*–*F*). The frequency of T_{FH} and GC B cells was also significantly reduced in *Etv5$^{f/f}$;Cd4-Cre* mice compared to that in WT mice following pristane treatment (Fig. 2 *G*–*I*). We also investigated whether ETV5 deficiency attenuated autoimmunity in *sanroque* mice, a lupus mouse model with an expanded T_{FH} cell population (37). T cell–specific deletion of *Etv5* alleles suppressed autoimmune-like manifestations such as increased serum Ig and anti-dsDNA antibody levels, tissue infiltration of immune cells, IgG deposition in kidney glomeruli, and T_{FH} and GC B cell formation in *sanroque* mice (*SI Appendix*, Fig. S3). Together, these data demonstrated that ETV5 is a transcription factor promoting T_{FH} cell–mediated lupus pathogenesis.

***Spp1* Is an ETV5 Target Gene Responsible for Promoting T_{FH} Cell Differentiation.** To understand how ETV5 promotes T_{FH} cell differentiation at the molecular level, we analyzed the gene expression profiles of WT and *Etv5*-null T_{FH} cells by RNA sequencing. In total, 92 genes were differentially expressed in *Etv5*-null T_{FH} cells (46 up-regulated and 46 down-regulated; *SI Appendix*, Table S1) compared to WT cells (fold-change > 2 and $P < 0.05$). Since ETV5 usually functions as a transcriptional activator, we primarily focused on the down-regulated genes in *Etv5*-null T_{FH} cells to identify the ETV5 target genes responsible for promoting T_{FH} cell differentiation. Among the genes down-regulated in *Etv5*-null T_{FH} cells, 10 were up-regulated in T_{FH} cells compared to non-T_{FH} cells, according to a previous report describing gene expression profiles in murine T_{FH} and non-T_{FH} cells (38) (*SI Appendix*, Fig. S4*A*). Among them, *Spp1*, *Tshz2*, and *Slc37a2* have a consensus ETV5 binding motif [5′-(C/G)(C/A)GGA(A/T)(G/C)(T/C)(G/A)-3′] within their promotor regions (upstream 1kb from the transcription start site) (39) (Fig. 3*A* and *SI Appendix*, Fig. S4*B*). Notably, the ETV5 binding motif in the *Spp1* promoter region is conserved in humans (*SI Appendix*, Fig. S4*C*). To examine whether these three genes were ETV5

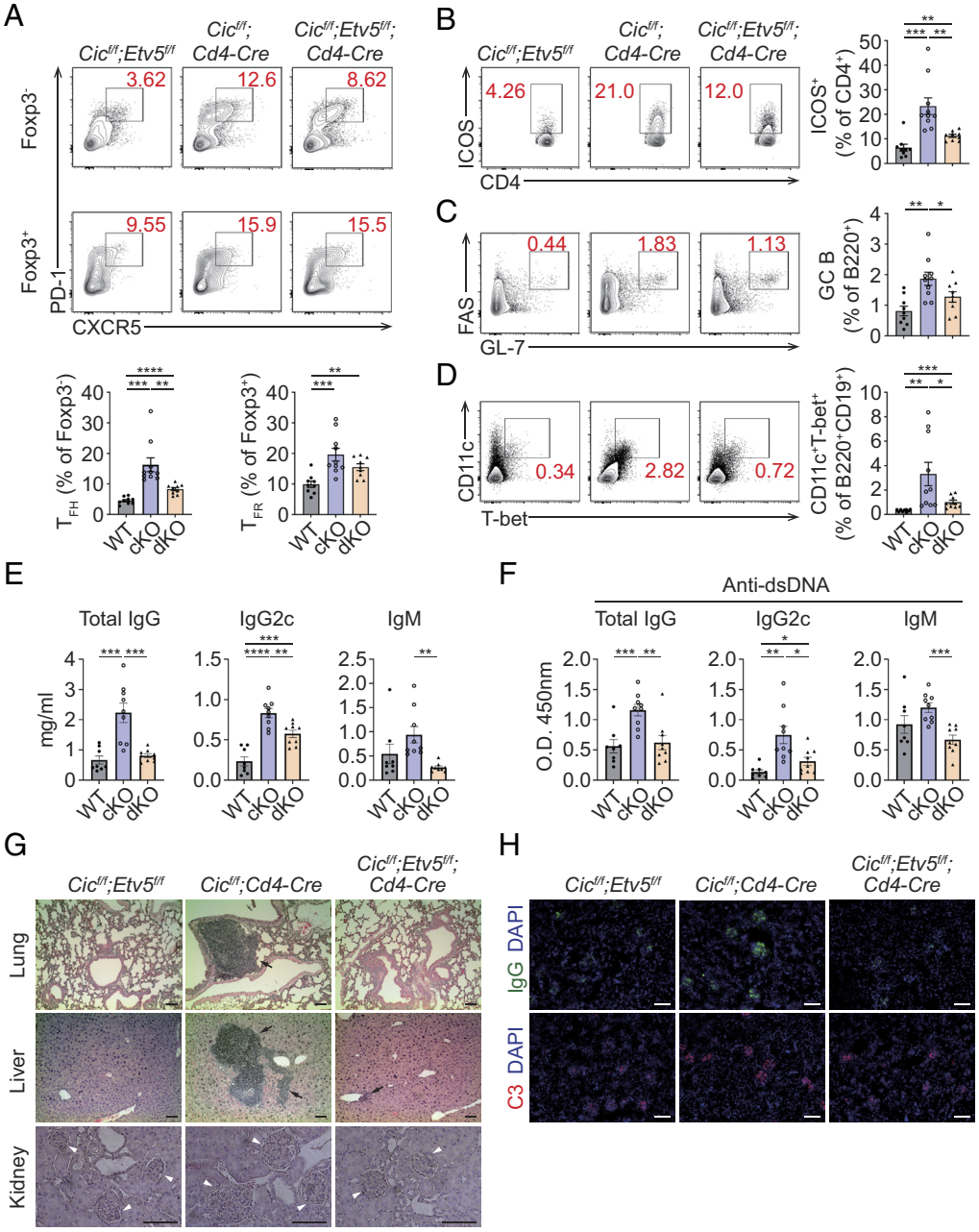

Fig. 1. Recovery of autoimmune-like phenotypes in T cell–specific *Cic* and *Etv5* double-null mice. (*A–D*) Flow cytometry of T_{FH} and T_{FR} cells (*A*), $CD4^+ICOS^+$ T cells (*B*), GC B cells (*C*), and $CD11c^+Tbet^+$ B cells (*D*) from spleens of 8-mo-old *Cic*$^{f/f}$;*Etv5*$^{f/f}$ ($n = 9$), *Cic*$^{f/f}$;*Cd4-Cre Cre* ($n = 10$), and *Cic*$^{f/f}$;*Etv5*$^{f/f}$;*Cd4-Cre* ($n = 9$) mice. Each dot in the graphs represents an individual mouse. These data represent the combination of results from three independent experiments. (*E* and *F*) Serum levels of total IgG, IgG2c, and IgM (*E*) and anti-dsDNA antibodies (*F*) in *Cic*$^{f/f}$;*Etv5*$^{f/f}$ ($n = 8$), *Cic*$^{f/f}$;*Cd4-Cre* ($n = 9$), and *Cic*$^{f/f}$;*Etv5*$^{f/f}$;*Cd4-Cre* ($n = 9$) mice as measured by the ELISA. The graphs show the data as mean ± SEM. Each dot in the graphs represents an individual mouse. WT: *Cic*$^{f/f}$;*Etv5*$^{f/f}$, cKO: *Cic*$^{f/f}$;*Cd4-Cre*, and dKO: *Cic*$^{f/f}$;*Etv5*$^{f/f}$;*Cd4-Cre*. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$. (*G*) Hematoxylin and eosin staining of the liver, lung, and kidney of 8-mo-old *Cic*$^{f/f}$;*Etv5*$^{f/f}$, *Cic*$^{f/f}$;*Cd4-Cre*, and *Cic*$^{f/f}$;*Etv5*$^{f/f}$;*Cd4-Cre* mice. Black arrows and white arrowheads indicate infiltrating immune cells and glomeruli, respectively. Two independent experiments were performed. (Scale bar, 100 μm.) (*H*) Representative immunofluorescence images of kidney sections of 8-mo-old *Cic*$^{f/f}$;*Etv5*$^{f/f}$, *Cic*$^{f/f}$;*Cd4-Cre*, and *Cic*$^{f/f}$;*Etv5*$^{f/f}$;*Cd4-Cre* mice. Kidney sections were stained with anti-IgG (green), anti-C3 (red), and DAPI (blue). Two independent experiments were performed. (Scale bar, 100 μm.)

targets, we performed chromatin immunoprecipitation (ChIP) using an anti-ETV5 antibody, followed by qPCR analysis of their promoter regions harboring the ETV5 binding motif. Chromatin was prepared from WT and *Etv5*-null $CD4^+$ T cells cultured under T_{FH}-like cell differentiation conditions (anti-CD3/CD28 plus IL-6, IL-21, anti-IL-4, anti-IFN-γ, and anti-TGF-β) for 3 d. Of the three genes, only the *Spp1* promoter region was significantly enriched in the chromatin immunoprecipitated using an anti-ETV5 antibody (Fig. 3*B*). To confirm that ETV5 activated *Spp1* transcription by binding to the ETV5 binding motif, we performed a dual-luciferase assay using a luciferase reporter plasmid containing the *Spp1* promoter region. ETV5 overexpression significantly increased the *Spp1* promoter activity; however, this effect was mitigated when the ETV5 binding motif was mutated (Fig. 3*C*). To validate the regulation of *Spp1* expression by ETV5, we compared *Spp1* levels between WT and *Etv5*-null $CD4^+$ T cells. *Spp1* levels were significantly decreased in *Etv5*-null $CD4^+$ naive T, T_{FH}, and follicular regulatory T (T_{FR}) cells compared to their respective control cells (Fig. 3*D*). Additionally, *Spp1* levels were significantly reduced in *Etv5*-null $CD4^+$ T cells compared to WT cells when cultured under T_{FH}-like cell differentiation conditions for 72 h (Fig. 3*E*).

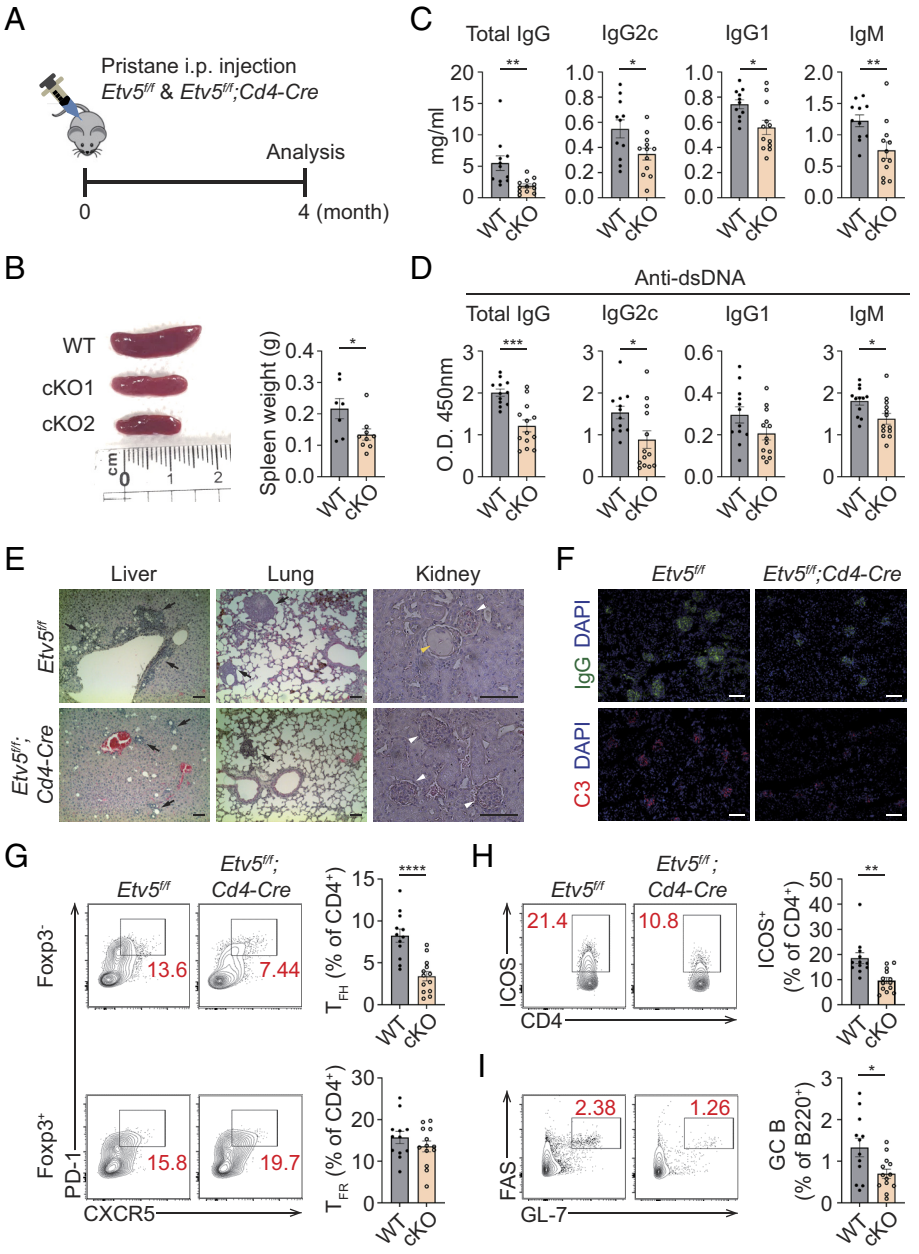

Fig. 2. Attenuation of pristane-induced autoimmune-like phenotypes by ETV5 deficiency. (*A*) Schematic illustration of the experiment. Four months after injecting pristane, mice were analyzed for autoimmune phenotypes and the frequency of T_{FH} and GC B cells. (*B*) Images of the spleens dissected from *Etv5*$^{f/f}$ and *Etv5*$^{f/f}$*;Cd4-Cre* mice treated with pristane for 4 mo. The graph shows the spleen weights of *Etv5*$^{f/f}$ (n = 7) and *Etv5*$^{f/f}$*;Cd4-Cre* (n = 9) mice treated with pristane. These data represent the combination of results from three independent experiments. (*C* and *D*) Immunoglobulin (*C*) and anti-dsDNA antibody (*D*) levels in the serum of *Etv5*$^{f/f}$ and *Etv5*$^{f/f}$*;Cd4-Cre* mice treated with pristane for 4 mo as measured by ELISA. Each dot in the graphs represents an individual mouse (immunoglobulins: WT, n = 11 and cKO, n = 12; anti-dsDNA antibody: WT, n = 12 and cKO, n = 13). (*E*) Hematoxylin and eosin staining of the liver, lung, and kidney sections from *Etv5*$^{f/f}$ and *Etv5*$^{f/f}$*;Cd4-Cre* mice treated with pristane for 4 mo. Black arrows indicate tissue infiltration of immune cells. The white and yellow arrowheads represent glomeruli and glomerular necrosis, respectively. Two independent experiments were performed. (Scale bar, 100 μm.) (*F*) Immunofluorescence images showing the deposition of IgG and C3 in the kidney glomeruli of *Etv5*$^{f/f}$ and *Etv5*$^{f/f}$*;Cd4-Cre* mice treated with pristane for 4 mo. Kidney sections were stained with anti-IgG (green), anti-C3 (red), and DAPI (blue). Two independent experiments were performed. (Scale bar, 100 μm.) (*G–I*) Flow cytometry showing the frequency of T_{FH} and T_{FR} cells (*G*), $CD4^+ICOS^+$ T cells (*H*), and GC B cells (*I*) in the spleens of *Etv5*$^{f/f}$ (n = 12) and *Etv5*$^{f/f}$*;Cd4-Cre* (n = 13) mice treated with pristane for 4 mo. The graphs show the data as mean ± SEM. Each dot in the graphs represents an individual mouse. These data represent the combination of results from five independent experiments. WT: *Etv5*$^{f/f}$ and cKO: *Etv5*$^{f/f}$*;Cd4-Cre*. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

We then examined whether the ETV5 deficiency–mediated attenuation of T_{FH} cell differentiation was due to reduced *Spp1* expression. WT and *Etv5*-null $Thy1.1^+$ OT-II cells infected with control or *Spp1*-overexpressing retroviruses were adoptively transferred into $Thy1.2^+$ recipient mice. Seven days after immunization with OVA in alum, donor OT-II cells were analyzed for T_{FH} cell differentiation using flow cytometry. *Spp1* overexpression promoted T_{FH} cell differentiation in WT OT-II cells (Fig. 3*F*). Moreover, *Spp1* overexpression completely rescued the defects in T_{FH} differentiation in *Etv5*-null OT-II cells (Fig. 3*F*). We established an in vitro mouse T_{FH} differentiation assay using OT-II cells (*SI Appendix*, Fig. S5*A*), following a previously reported method (40), and confirmed these results in vitro as well (*SI Appendix*, Fig. S5*B*). Interestingly, similar results were also observed in GFP^- OT-II cells, which were not infected with control or *Spp1*-overexpressing retroviruses (*SI Appendix*, Fig. S5*C*). Overall, our data indicated that *Spp1* is an ETV5 target gene responsible for promoting T_{FH} cell differentiation.

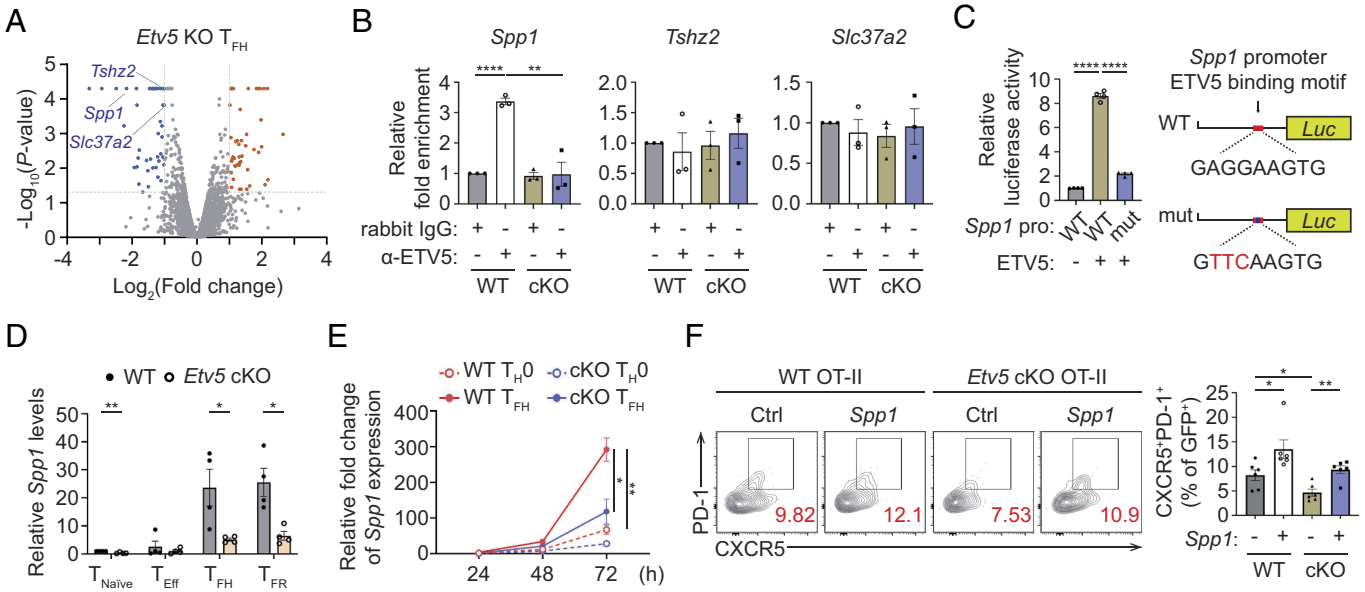

Fig. 3. *Spp1* as an ETV5 target gene that promotes T_{FH} cell differentiation. (*A*) Volcano plot showing differentially expressed genes (DEGs) in *Etv5*-null T_{FH} cells. (*B*) ChIP-qPCR analysis showing ETV5 occupancy of the *Spp1* promoter in $CD4^+$ T cells. qPCR analysis was conducted for the *Spp1*, *Tshz2*, and *Slc37a2* promoter regions containing the ETV5 binding motif. Three independent experiments were performed. (*C*) A dual luciferase assay showing that ETV5 enhances *Spp1* promoter activity through the ETV5 binding motif ($n = 4$ per group, combined from two independent experiments). The *Right* panel shows a mutation of the ETV5 binding motif in the *Spp1* promoter. (*D*) qRT-PCR analysis of *Spp1* levels in naive ($CD4^+CD44^-CXCR5^-PD\text{-}1^-$), effector ($CD4^+CD44^+CXCR5^-PD\text{-}1^-$), T_{FH} ($CD4^+CXCR5^+PD\text{-}1^+GITR^-$), and T_{FR} ($CD4^+CXCR5^+PD\text{-}1^+GITR^+$) cells from *Etv5*$^{f/f}$ and *Etv5*$^{f/f}$*;Cd4-Cre* mice. Four independent experiments were performed. (*E*) qRT-PCR analysis for *Spp1* levels. Naive $CD4^+$ T cells isolated from the spleens of *Etv5*$^{f/f}$ and *Etv5*$^{f/f}$*;Cd4-Cre* mice were activated with anti-CD3 and anti-CD28 and cultured in the presence (T_{FH}) or absence (T_H0) of IL-6 and IL-21 for 24, 48, and 72 h. Three independent experiments were conducted. (*F*) Adoptive transfer experiments using $Thy1.1^+$ WT and $Thy1.1^+$ *Etv5*-null OT-II cells infected with control or *Spp1*-expressing retrovirus ($n = 6$ per group, combined from two independent experiments). The transferred GFP^+ OT-II cells were analyzed for T_{FH} cell differentiation. The bar graphs show the data as mean ± SEM. WT: *Etv5*$^{f/f}$ and cKO: *Etv5*$^{f/f}$*;Cd4-Cre*. $^*P < 0.05$, $^{**}P < 0.01$, and $^{****}P < 0.0001$.

Secreted OPN Promotes T_{FH} Cell Differentiation by Activating the CD44-AKT Pathway. *Spp1* encodes OPN, a secreted, phosphorylated glycoprotein that mediates diverse biological functions (41). Dysregulated OPN expression is associated with the pathogenesis of autoimmune diseases such as rheumatoid arthritis and SLE (42, 43). An intracellular form of OPN (OPN-i) is produced by translation initiation downstream of the start codon of *Spp1* (44). As OPN-i is known to interact with and stabilize BCL6, the lineage-defining transcription factor for T_{FH} cell differentiation (13–15), to ensure the continued development and function of T_{FH} cells (45), we measured OPN and BCL6 levels in WT and *Etv5*-null T cells. The levels of intracellular OPN were comparable between WT and *Etv5*-null T_{FH} cells (*SI Appendix*, Fig. S6*A*), whereas OPN secretion was lower in *Etv5*-null T_{FH} cells compared to WT cells (*SI Appendix*, Fig. S6*B*). BCL6 levels in T_{FH} cells were comparable among the WT, *Cic*$^{f/f}$*;Cd4-Cre*, and *Cic*$^{f/f}$*;Etv5*$^{f/f}$*;Cd4-Cre* mice (*SI Appendix*, Fig. S6*C*). Moreover, the levels of BCL6 in T_{FH} cells were comparable between WT and *Etv5*$^{f/f}$*;Cd4-Cre* mice, regardless of whether they were subjected to OVA immunization or pristane treatment (*SI Appendix*, Fig. S6 *D* and *E*). Overall, these data demonstrated that the attenuation of T_{FH} cell differentiation by ETV5 deficiency was not due to a defect in OPN-i expression.

Given that treatment with the *Spp1*-overexpressing retrovirus promotes T_{FH} cell differentiation not only in infected cells but also in uninfected cells (*SI Appendix*, Fig. S5*C*), we explored the possibility that secreted OPN could promote T_{FH} cell differentiation. Blocking protein secretion by treatment with Golgi-Stop and Golgi-Plug dramatically increased intracellular OPN levels in $CD4^+$ T cells cultured under T_{FH}-like cell differentiation conditions (Fig. 4*A*), suggesting that most of the OPN produced by T_{FH} cells is secreted. In line with the previous finding (*SI Appendix*, Fig. S6*B*), a greater amount of OPN accumulated in WT cells than in *Etv5*-deficient cells when treated with Golgi-Stop and Golgi-Plug (Fig. 4*A*), confirming that *Etv5*-deficient T_{FH} cells secrete less OPN than WT T_{FH} cells. To investigate the effect of extracellular OPN on T_{FH} cell differentiation, we performed an in vitro mouse T_{FH} cell differentiation assay using OT-II cells. Treatment with recombinant mouse OPN significantly enhanced the efficiency of T_{FH} cell differentiation (Fig. 4*B*). In contrast, treatment with recombinant OPN (1 ng/mL) did not enhance differentiation of naive $CD4^+$ T cells into T_H1, T_H2, T_H17, or T_{REG} cells in vitro (*SI Appendix*, Fig. S7*A*). Because secreted OPN activates the PI3K-AKT pathway (46), important for T_{FH} cell differentiation (47, 48), we performed the same experiments in the presence of an AKT inhibitor (MK2206). AKT inhibition abolished the T_{FH} cell differentiation-promoting effect of OPN (Fig. 4*B*), suggesting that extracellular OPN promotes T_{FH} cell differentiation by activating AKT.

OPN binds to integrins and CD44 to activate the PI3K-AKT pathway (46). To investigate which OPN receptor is responsible for promoting T_{FH} cell differentiation, we conducted in vitro mouse T_{FH} cell differentiation assays using OT-II cells in the presence or absence of blocking antibodies against integrins and CD44. Treatment with an anti-CD44 antibody significantly inhibited the T_{FH} cell differentiation-promoting effect of OPN, whereas treatment with either anti-integrin αV or anti-integrin β3 antibody did not exhibit this effect (Fig. 4*C*). Since the Thr137 and Ser147 residues of mouse OPN are essential for the activation of the CD44 receptor (49), we examined the activity of mutant OPNs with alanine substitutions at the Thr137 (T137A) or Ser147 (S147A) residues on T_{FH} cell differentiation by performing adoptive transfer experiments and in vitro mouse T_{FH} cell differentiation assays using OT-II cells. Overexpression of the T137A and S147A mutants did not affect T_{FH} cell differentiation, whereas overexpression of WT OPN promoted this process (Fig. 4*D* and

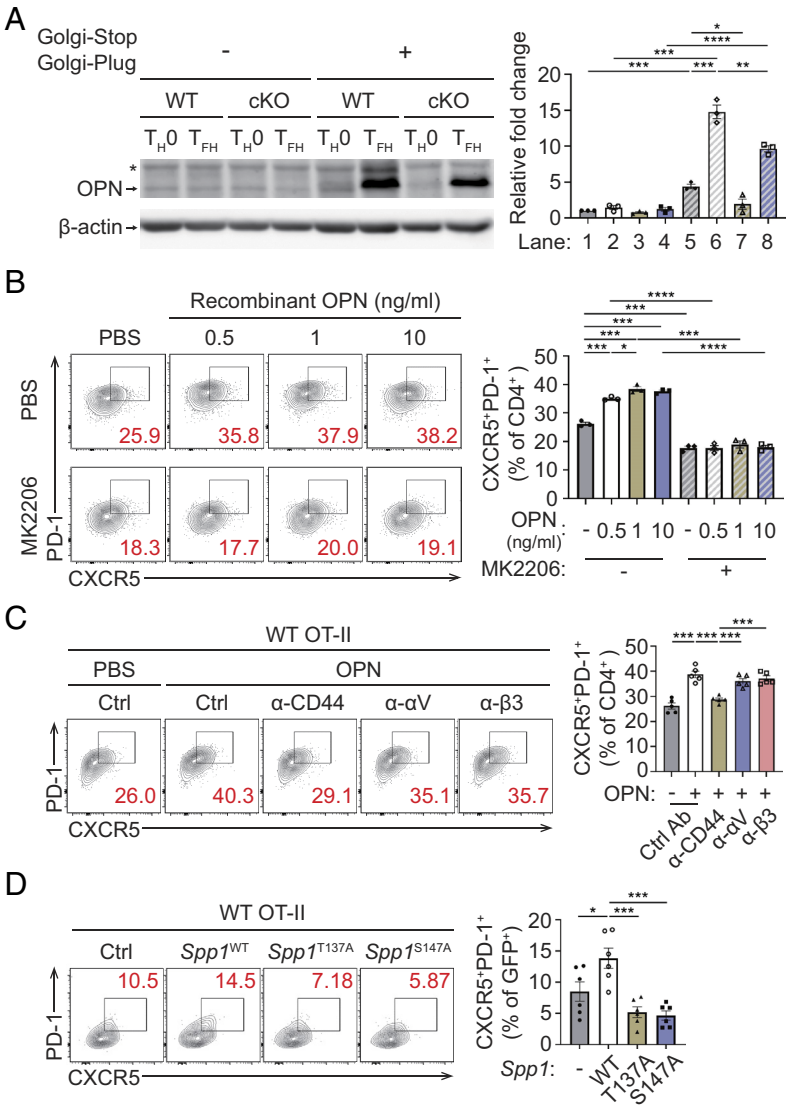

Fig. 4. Promotion of T_{FH} cell differentiation by extracellular OPN. (*A*) Western blotting for the levels of OPN in naive $CD4^+$ T cells isolated from the spleens of *Etv5*$^{f/f}$ and *Etv5*$^{f/f}$*;Cd4-Cre* mice, activated with anti-CD3 and anti-CD28, and cultured in the presence (T_{FH}) or absence (T_H0) of IL-6 and IL-21 for 72 h, followed by culturing in the presence or absence of Golgi-Stop and Golgi-Plug for 4 h. The asterisk indicates a nonspecific band. The bar graph shows the relative levels of OPN. Three independent experiments were conducted. WT: *Etv5*$^{f/f}$ and cKO: *Etv5*$^{f/f}$*;Cd4-Cre*. (*B*) Flow cytometry for the frequency of $CXCR5^+PD\text{-}1^+$ T_{FH} cells in WT OT-II cells cultured under T_{FH} polarizing conditions in the presence or absence of recombinant mouse OPN and MK2206 AKT inhibitor (1 μM) for 3 d (n = 3 per group). Two independent experiments were performed. (*C*) Flow cytometry for the frequency of T_{FH} cells in WT OT-II cells cultured under T_{FH}-polarizing conditions in the presence or absence of recombinant mouse OPN (1 ng/mL) and control antibody (Rat IgG1) (10 μg/mL), or blocking antibodies to CD44 (10 μg/mL), integrin αV (10 μg/mL), or integrin β3 (10 μg/mL) for 3 d (n = 5 per group, combined from two independent experiments). (*D*) Flow cytometry of T_{FH} cell differentiation in $Thy1.1^+$ OT-II cells infected with control, *Spp1*^{WT}-, *Spp1*^{T137A}-, or *Spp1*^{S147A}-expressing retrovirus, adoptively transferred to $Thy1.2^+$ B6 recipient mice, and immunized with OVA for 7 d (n = 6 per group, combined from two independent experiments). The bar graphs present the data as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

SI Appendix, Fig. S5*D*). Consistent with the previous finding (*SI Appendix*, Fig. S5*C*), differentiation into T_{FH} cells was also promoted in GFP^- OT-II cells in vitro by overexpression of WT OPN, while this effect was not observed when the T137A and S147A OPN mutants were overexpressed (*SI Appendix*, Fig. S5*E*). Taken together, these data indicate that OPN promotes T_{FH} cell differentiation by binding to CD44.

Recombinant OPN Treatment Recovers T_{FH} and GC B Cell Formation in *Etv5*$^{f/f}$*;Cd4-Cre* Mice. To determine whether the reduced secretion of OPN attenuated T_{FH} cell differentiation in *Etv5*-null $CD4^+$ T cells, we performed an in vitro mouse T_{FH} cell differentiation assay using WT and *Etv5*-null OT-II cells. Recombinant OPN treatment completely restored T_{FH} cell differentiation efficiency of *Etv5*-null OT-II cells (*SI Appendix*, Fig. S5*F*). To validate this result in vivo, WT and *Etv5*$^{f/f}$*;Cd4-Cre* mice were intravenously injected with recombinant mouse OPN, followed by immunization with OVA in alum, and a second intravenous injection of recombinant mouse OPN (Fig. 5*A*). Seven days after OVA immunization, we examined the frequency of T_{FH} and GC B cells and the level of phospho-AKT in T_{FH} cells using flow cytometry (Fig. 5*A*). Treatment with recombinant mouse OPN promoted T_{FH} cell differentiation in WT mice and completely restored the frequency of T_{FH} cells to WT levels in *Etv5*$^{f/f}$*;Cd4-Cre* mice (Fig. 5*B*). Consistent with this result, GC B cell frequency was rescued in *Etv5*$^{f/f}$*;Cd4-Cre* mice treated with recombinant OPN (Fig. 5*C*). Moreover, the decreased phospho-AKT levels were significantly restored in *Etv5*-null T_{FH} cells after treatment with recombinant OPN (Fig. 5*D*). In contrast, OPN treatment did not affect T_H1, T_H2, T_H17, and T_{REG} cell formation

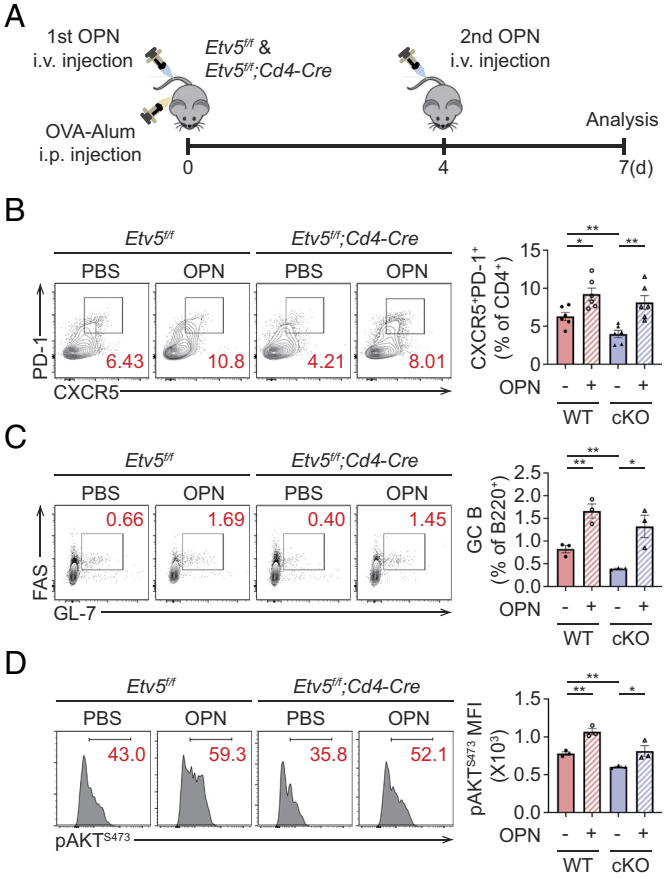

Fig. 5. Recovery of T_{FH} and GC B cell formation in T cell–specific *Etv5*-null mice by recombinant OPN treatment. (*A*) Schematic illustration of the experiment. Mice were analyzed for the frequency of T_{FH} and GC B cells and the level of $pAKT^{S473}$ in T_{FH} cells. (*B* and *C*) Flow cytometry for the frequency of T_{FH} (*B*) and GC B (*C*) cells from the spleens of $Etv5^{f/f}$ and $Etv5^{f/f}$;*Cd4-Cre* mice treated with recombinant mouse OPN (T_{FH}: $n = 6$ per group, combined from two independent experiments; GC B: $n = 3$ per group). (*D*) Quantification of $pAKT^{S473}$ mean fluorescence intensity (MFI) in T_{FH} cells from $Etv5^{f/f}$ and $Etv5^{f/f}$;*Cd4-Cre* mice treated with recombinant mouse OPN ($n = 3$ per group). The bar graphs present the data as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

in WT mice (*SI Appendix*, Fig. S7*B*), which is consistent with the results of the in vitro T_H subset differentiation assays (*SI Appendix*, Fig. S7*A*). Overall, these data demonstrate that ETV5 promotes T_{FH} cell differentiation through OPN-mediated activation of the CD44-AKT pathway.

The Molecular Mechanism Underlying the Regulation of T_{FH} Cell Differentiation by ETV5 Is Conserved in Humans. We sought to determine whether the regulatory roles of ETV5 and OPN in T_{FH} cell differentiation were conserved in humans. We compared *ETV1*, *ETV4*, *ETV5*, and *SPP1* levels in different subsets of human $CD4^+$ T cells. *ETV5* and *SPP1* levels were significantly higher in T_{FH} cells than those in naive and effector $CD4^+$ T cells, whereas *ETV1* and *ETV4* levels were not significantly altered (Fig. 6*A*). The expression of *ETV5* and *SPP1* was significantly up-regulated in human $CD4^+$ T cells cultured under T_{FH} cell differentiation conditions (anti-CD3/CD28 plus IL-12 and TGF-β) for 3 d (Fig. 6*B*). This result was also validated at the protein level (Fig. 6*C*). We also confirmed that *SPP1* is an ETV5 target gene in humans by performing a dual-luciferase assay using human *SPP1* promoter-containing luciferase reporter plasmids (Fig. 6*D*). We then determined whether changes in *ETV5* and *SPP1* levels affected human T_{FH} cell differentiation. Overexpression of *ETV5* increased the frequency of T_{FH} cells in T_H0 conditions (anti-CD3/CD28 only), as well as in T_{FH} cell differentiation conditions (Fig. 6*E*). Knockdown of *ETV5* attenuated T_{FH} cell differentiation (Fig. 6*F*). Overexpression and knockdown of *SPP1* had similar effects on human T_{FH} cell differentiation (Fig. 6 *G* and *H*).

Finally, we examined whether the secreted OPN promotes human T_{FH} cell differentiation by activating the CD44-AKT pathway. Treatment with recombinant human OPN enhanced T_{FH} cell differentiation in vitro, whereas this effect was abolished by AKT inhibition (Fig. 6*I*). Mutation of the T152 and S162 residues of human OPN, which correspond to T137 and S147 of mouse OPN, respectively (49), and the inhibition of CD44 binding to OPN by anti-CD44 antibody treatment abolished the T_{FH} cell differentiation-promoting effect of OPN (Fig. 6 *J* and *K*). Taken together, like in mice, ETV5 promotes T_{FH} cell differentiation in humans through OPN-mediated activation of the CD44-AKT pathway.

Disease Activity in Patients with SLE Is Associated with *ETV5* and *SPP1* Levels in $CD4^+$ T Cells. Finally, we analyzed samples from patients with SLE to gain clinical insights into the regulation of SLE progression by the ETV5-*SPP1* axis. We measured *ETV5* and *SPP1* levels in peripheral blood $CD4^+$ T cells from healthy individuals and patients with SLE with different disease severities using qRT-PCR (*SI Appendix*, Table S2). As expected, *ETV5* levels significantly correlated with *SPP1* levels in $CD4^+$ T cells from healthy individuals and patients with SLE (Fig. 7*A*). Importantly, the levels of *ETV5* and *SPP1* in $CD4^+$ T cells were significantly higher in SLE patients than in healthy individuals (Fig. 7*B*). This observation was not solely due to an increase in the frequency of circulating T_{FH} cells in patients with SLE, as there was no correlation between the expression of T_{FH} specific genes such as *BCL6* and *ICOS*, and that of *ETV5* or *SPP1* in individual samples (*SI Appendix*, Fig. S8). We further analyzed the association between *ETV5* and *SPP1* levels and disease activity in 25 patients with SLE (*SI Appendix*, Table S2). *ETV5* and *SPP1* levels in $CD4^+$ T cells significantly correlated with SLE disease activity index (SLEDAI) and serum anti-dsDNA antibody levels in patients with SLE (Fig. 7 *C* and *D*). Given that the overexpression of *ETV5* and *SPP1* in $CD4^+$ T cells enhanced human T_{FH} cell differentiation (Fig. 6 *E* and *G*), these data suggest a potential role for the ETV5-*SPP1* axis in regulating T_{FH} cell–mediated lupus pathogenesis in humans.

Discussion

In this study, we investigated the role of ETV5 in T_{FH} cell differentiation and lupus pathogenesis in mice and humans. ETV5 is a transcription factor that promotes the pathogenesis of lupus by enhancing T_{FH} cell differentiation. Our previous study showed that ETV5 derepression up-regulates the expression of *Maf* in *Cic*-deficient T_{FH} cells, and the knockdown of *Maf* attenuates T_{FH} cell differentiation in *Cic*-deficient OT-II cells (34). Based on these results, we proposed that *Maf* was an ETV5 target gene involved in CIC deficiency–mediated promotion of T_{FH} cell differentiation (34). However, RNA sequencing revealed that *Maf* levels were not significantly reduced in *Etv5*-null T_{FH} cells (*SI Appendix*, Table S1). These results indicate that ETV5 loss is insufficient to down-regulate the expression of *Maf*, implying that other ETS transcription factors, such as ETV1 and ETV4, might compensate for the loss of ETV5 to maintain *Maf* expression in T_{FH} cells.

Through RNA sequencing and molecular and cellular biology approaches, we identified *SPP1* as an ETV5 target gene important for regulating T_{FH} cell differentiation in mice and humans. Several studies have shown that *Spp1* deficiency suppresses T_{FH} differentiation

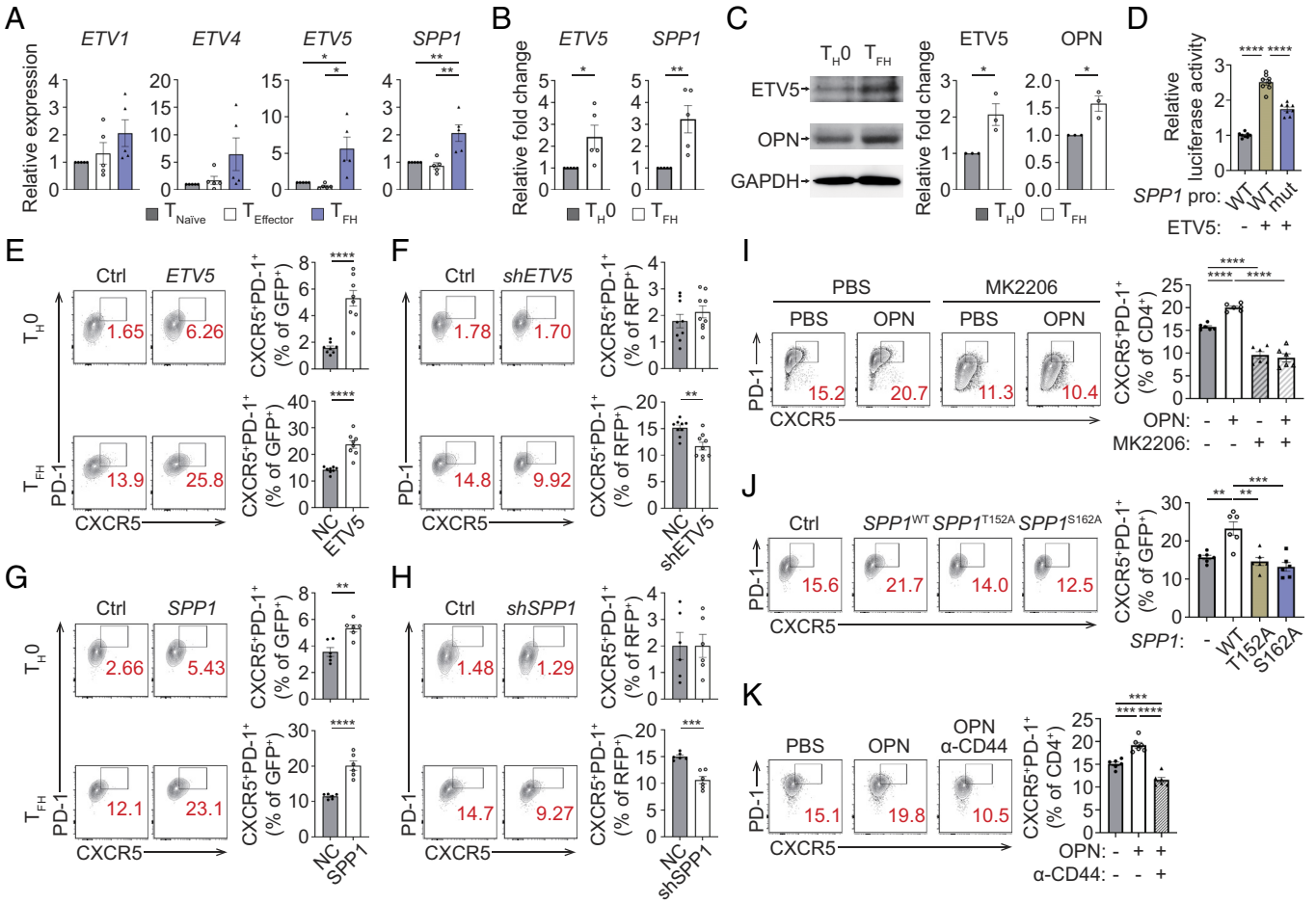

Fig. 6. Regulation of T_{FH} cell differentiation by ETV5 and extracellular OPN in humans. (*A*) qRT-PCR analysis for the levels of *ETV1*, *ETV4*, *ETV5*, and *SPP1* in naive ($CD4^+CD45RA^+CXCR5^-PD\text{-}1^-$), effector ($CD4^+CD45RA^-CXCR5^-PD\text{-}1^-$), and T_{FH} ($CD4^+CD45RA^-CXCR5^+PD\text{-}1^+$) cells sorted from human tonsils (n = 5 per group). Five independent experiments were performed. (*B*) qRT-PCR analysis of *ETV5* and *SPP1* expression in human $CD4^+$ T cells cultured for 3 d under T_H0- or T_{FH}-polarizing conditions (n = 5 per group). Five independent experiments were performed. (*C*) Western blotting for the levels of ETV5 and OPN in human $CD4^+$ T cells cultured for 3 d under T_H0- or T_{FH}-polarizing conditions. The bar graphs show the relative levels of ETV5 and OPN. Three independent experiments were performed. (*D*) Dual luciferase assay showing that ETV5 enhances *SPP1* promoter activity through the ETV5 binding motif (n = 8 per group). These data represent the combination of results from two independent experiments. (*E–H*) Flow cytometry for the regulation of human T_{FH} cell differentiation by *ETV5* and *SPP1*. Human $CD4^+$ T cells transduced with *ETV5*- (n = 8 per group, combined from three independent experiments) (*E*), *shETV5*- (n = 9 per group, combined from three independent experiments) (*F*), *SPP1*- (n = 6 per group, combined from two independent experiments) (*G*), and *shSPP1*- (n = 6 per group, combined from two independent experiments) (*H*) expressing retroviruses were cultured under T_H0- or T_{FH}-polarizing conditions for 3 d. GFP^+ cells (*E* and *G*) or RFP^+ cells (*F* and *H*) in each condition were analyzed for the frequency of $CXCR5^+PD\text{-}1^+$ T_{FH} cells by flow cytometry. (*I*) Flow cytometry of human $CD4^+$ T cells cultured under T_{FH}-polarizing conditions in the presence or absence of recombinant human OPN (1 ng/mL) and MK2206 AKT inhibitor (1 μM) (n = 6 per group, combined from two independent experiments). (*J*) Flow cytometry for the frequency of $CXCR5^+PD\text{-}1^+$ T_{FH} cells in human $CD4^+$ T cells transduced with control, $SPP1^{WT}$-, $SPP1^{T152A}$-, and $SPP1^{S162A}$-expressing retroviruses and cultured under T_{FH}-polarizing conditions for 3 d (n = 6 per group, combined from two independent experiments). (*K*) Flow cytometry for the frequency of $CXCR5^+PD\text{-}1^+$ T_{FH} cells in human $CD4^+$ T cells cultured for 3 d under T_{FH}-polarizing conditions in the presence or absence of recombinant human OPN (1 ng/mL) and CD44 neutralizing antibody (10 μg/mL) (n = 6 per group, combined from two independent experiments). The bar graphs present the data as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

(42, 45). Leavenworth et al. found that inducing OPN-i expression through the Cre-mediated deletion of the floxed stop cassette restores defects in T_{FH} cell formation in *Spp1*-deficient mice (45). They also showed that OPN-i interacted with and stabilized BCL6 in the nucleus, enhancing T_{FH} cell differentiation and function (45). However, we found that the levels of intracellular OPN and BCL6 were not reduced in *Etv5*-null T_{FH} cells. This unexpected result led us to uncover a unique mechanism by which secreted OPN promotes T_{FH} cell differentiation. Since most of the OPN produced in T_{FH}-like cells was secreted (Fig. 4*A*), secreted OPN may have a dominant effect on promoting T_{FH} cell differentiation compared to OPN-i. Given that excessive formation of T_{FH} cells and increased serum OPN levels are often found in lupus mouse models and in patients with SLE (50–52), our findings explain how these two phenomena are linked at the molecular level in the context of lupus. While the concentration of OPN in the sera of SLE patients positively correlates with disease activity (53–56), the mechanism by which serum OPN levels increase in patients with SLE remains unclear. To better understand the pathogenesis of SLE, in-depth studies on the regulation of extracellular OPN levels and the impact of dysregulated extracellular OPN levels on T_{FH} cell formation in patients with SLE and mouse models are required.

OPN is known to regulate the differentiation of several T_H subsets (57). In vitro treatment with 1 μg/mL of recombinant mouse OPN significantly enhances T_H1 and T_H17 cell differentiation and inhibits IL-10 expression in T cells (58). However, treatment with 1 ng/mL recombinant mouse OPN did not affect T_H1, T_H2, T_H17, or T_{REG} cell differentiation in vitro (*SI Appendix*, Fig. S7*A*), while T_{FH} cell differentiation was significantly enhanced (Fig. 4*B*). Furthermore, intravenous injection of recombinant OPN promoted T_{FH} cell differentiation without affecting the differentiation of other T_H subsets (Fig. 5*B* and *SI Appendix*, Fig. S7*B*). These data suggest that the differentiation of T_{FH} cells is more sensitively regulated by secreted OPN than the differentiation of other types of T_H cells.

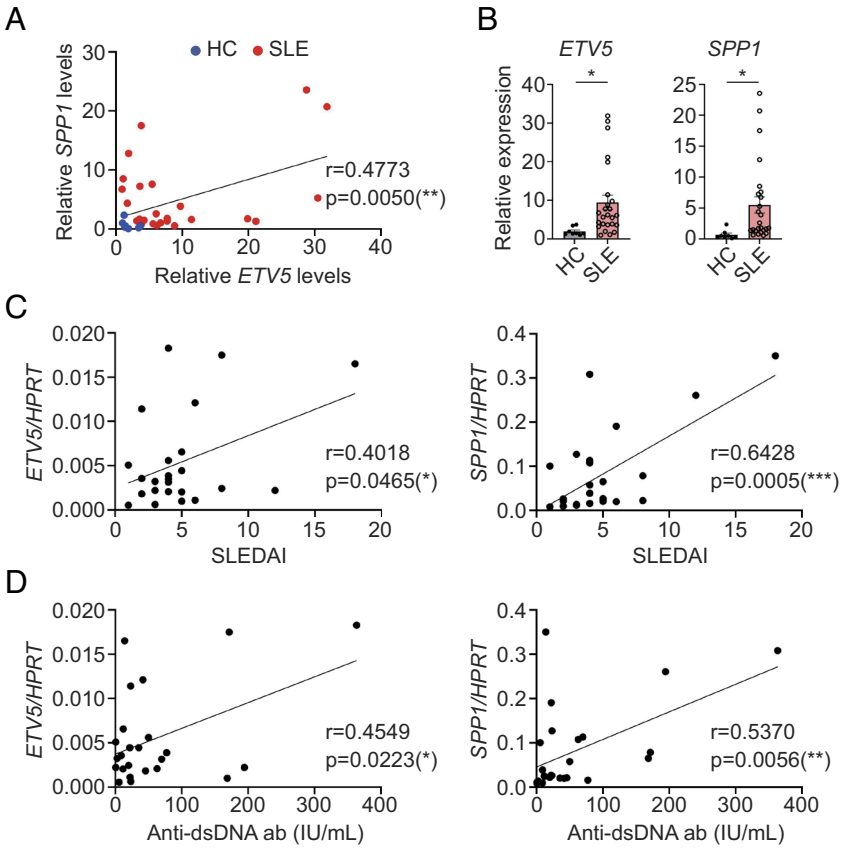

Fig. 7. Association of *ETV5* and *SPP1* levels in CD4$^+$ T cells with SLE disease activity. (*A*) The correlation between *ETV5* and *SPP1* expression in peripheral blood CD4$^+$ T cells obtained from healthy controls (HCs) (n = 8) and SLE patients (n = 25). The blue and red dots represent individual HC and SLE patients, respectively. (*B*) qRT-PCR analysis of *ETV5* and *SPP1* expression in peripheral blood CD4$^+$ T cells obtained from HCs (n = 8) and SLE patients (n = 25). The bar graphs present the data as mean ± SEM. (*C* and *D*) Positive correlation of *ETV5* and *SPP1* levels in CD4$^+$ T cells of SLE patients (n = 25) with SLEDAI score (*C*) and serum anti-dsDNA antibody levels (*D*). *P < 0.05, **P < 0.01, and ***P < 0.001.

The PI3K-AKT pathway plays a pivotal role in T_{FH} differentiation by regulating FOXO1, which inhibits *Bcl6* expression. Phosphorylation by AKT prompts nuclear export and cytoplasmic degradation of FOXO1, facilitating T_{FH} differentiation through the upregulation of BCL6 expression (48, 59). However, our study found that BCL6 levels were comparable between WT and *Etv5*-null T_{FH} cells, suggesting that AKT activation via OPN-CD44 signaling may not significantly affect FOXO1-mediated regulation of BCL6 expression. When mTORC2, a major player in AKT phosphorylation, is deficient in activated T cells, T_{FH} cell differentiation is significantly impaired without apparent changes in BCL6 levels in T_{FH} cells (60). The mTORC2-AKT pathway promotes glucose uptake and glycolysis, which are essential for T_{FH} cell differentiation (60, 61). Notably, OPN has been shown to enhance glycolysis in various cells (62), while the removal of CD44 leads to a reduction in glycolytic phenotypes (63, 64). Thus, activation of the PI3K-AKT pathway by OPN-CD44 signaling may promote T_{FH} cell differentiation by enhancing glucose metabolism.

Although our studies demonstrated that OPN expressed in T_{FH} cells plays an important role in T_{FH} cell differentiation, it remains unclear whether OPNs secreted by other cell types also contribute to this. Recombinant OPN treatment promoted T_{FH} cell differentiation both in vitro and in vivo, demonstrating that secreted OPN promotes T_{FH} cell differentiation in a paracrine manner. Because follicular DCs and B cells interact with developing T_{FH} cells (9), studying how OPNs secreted by DCs and B cells critically contribute to T_{FH} cell differentiation in secondary lymphoid organs would be interesting. Peripheral T helper (T_{PH}, also known as extrafollicular T helper) cells, which share many B helper-associated features with T_{FH} cells in terms of surface markers and gene expression profiles, are found in the tertiary lymphoid structures of inflamed tissues and have been implicated in the pathogenesis of autoimmune diseases and cancer (65, 66). Because OPN is expressed by various types of immune cells recruited to inflamed tissues, secreted OPN may promote the formation of T_{PH} cells, which express higher levels of CD44 than T_{FH} cells (66), in inflamed tissues. Further studies on the effects of secreted OPN on the differentiation of T_{FH} and T_{PH} cells in a disease context will contribute to developing treatment modalities for various immune disorders, including autoimmune diseases and cancers.

Materials and Methods

All materials and methods used in this study can be found in *SI Appendix, Materials and Methods*. All animal procedures were approved by the Institutional Animal Care and Use Committee of Pohang University of Science and Technology. The experimental procedures using human tonsil tissue and blood samples of healthy donors and SLE patients were approved by the Institutional Review Board of Seoul St. Mary's Hospital of the Catholic University of Korea (Human tonsil tissue: KC18TESI0723, blood samples: XC23TNDI0055).

Data, Materials, and Software Availability. Raw and processed RNA sequencing data generated in this study are available in the Gene Expression Omnibus (GEO) data repository under accession number GSE247030 (67). All other data are included in the manuscript and/or *SI Appendix*.

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