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Liver X receptor controls follicular helper T cell differentiation via repression of TCF-1

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Liver X receptor (LXR) is a critical regulator of cholesterol homeostasis that inhibits T cell receptor (TCR)-induced proliferation by altering intracellular sterol metabolism. However, the mechanisms by which LXR regulates helper T cell subset differentiation remain unclear. Here, we demonstrate that LXR is a crucial negative regulator of follicular helper T (Tfh) cells in vivo. Both mixed bone marrow chimera and antigen-specific T cell adoptive cotransfer studies show a specific increase in Tfh cells among LXRβ-deficient CD4$^+$ T cell population in response to immunization and lymphocytic choriomeningitis mammarenavirus (LCMV) infection. Mechanistically, LXRβ-deficient Tfh cells express augmented levels of T cell factor 1 (TCF-1) but comparable levels of Bcl6, CXCR5, and PD-1 in comparison with those of LXRβ-sufficient Tfh cells. Loss of LXRβ confers inactivation of GSK3β induced by either AKT/Extracellular signal-regulated kinase (ERK) activation or Wnt/β-catenin pathway, leading to elevated TCF-1 expression in CD4$^+$ T cells. Conversely, ligation of LXR represses TCF-1 expression and Tfh cell differentiation in both murine and human CD4$^+$ T cells. LXR agonist significantly diminishes Tfh cells and the levels of antigen-specific IgG upon immunization. These findings unveil a cell-intrinsic regulatory function of LXR in Tfh cell differentiation via the GSK3β-TCF1 pathway, which may serve as a promising target for pharmacological intervention in Tfh-mediated diseases.

liver X receptor | Tfh cells | TCF-1 | GSK3β | humoral immunity

Significance

Tfh cells are indispensable for effective humoral immunity against infection but detrimental in autoimmune diseases. We demonstrate that LXR, an oxysterol sensor previously known to integrate metabolic and inflammatory signaling, acts as a critical negative regulator of Tfh cell differentiation. LXR deficiency selectively and cell-intrinsically increases Tfh cell differentiation in immunization and viral infection models. Mechanistically, LXR suppresses TCF-1, a transcription factor required for the early Tfh lineage program, by regulating both TCR-mediated and Wnt/β-catenin-mediated inactivation of GSK3β. Since LXR also suppresses human Tfh cell differentiation, our findings accentuate the possibility of targeting LXR to treat Tfh cell-related diseases in humans.

Upon antigenic stimulation, antigen-specific T cells undergo clonal expansion to increase the proportion of antigen-specific T cells in the secondary lymphoid organs (1). After multiple rounds of division, activated T cells lose proliferative potential as a negative feedback mechanism (2). During the clonal expansion, activated T cells reprogram their bioenergetic metabolism to meet the high demands for substrates required for the clonal expansion (3). Anabolic reprogramming of lipid metabolism is necessary for activated T cells to support their cellular growth (4). In particular, cholesterol biosynthesis is up-regulated in the proliferating T cells as it is an essential component of cellular membranes (5). Cholesterol constitutes about 30% of cell membranes and plays an important role in maintaining the fluidity of the membrane (6). T cells are activated by forming an immunological synapse with antigen-presenting cells, during which cholesterol-rich lipid rafts are reorganized (7). In addition, since various receptors and signaling molecules exist in the cell membrane of T cells, cholesterol can regulate receptor signaling through membrane receptor localization and conformational change (8). For instance, hypercholesterol enhances TCR signaling and Treg cell formation (9, 10). Therefore, dynamic changes in cholesterol biosynthesis and metabolism occur during T cell activation and differentiation.

Homeostasis of intracellular cholesterol is regulated by the reciprocal activation of sterol regulatory element-binding proteins and LXRs (11). There exist two isoforms of LXR; LXRα is expressed in metabolically active tissues including the liver and adipose tissues, while LXRβ is ubiquitously expressed. LXRs function as sensors of oxysterols and sterol intermediates that are activated in response to elevated intracellular cholesterol levels. Once activated, LXR/Retionid X receptor (RXR) heterodimers induce the expression of an array of genes involved in cholesterol absorption and efflux as well as fatty acid metabolism (11). In addition to their function in lipid metabolism, LXRs modulate the differentiation and functions of diverse immune cells. For instance, LXRs suppress proinflammatory gene expression in macrophages and dendritic cells by suppressing nuclear factor kappa B activity (12, 13). LXR-deficient mice develop lupus-like phenotypes due to defective clearance of apoptotic bodies by macrophages (14). LXRβ-deficient T cells show increased proliferation upon TCR stimulation in vitro as well as in response to viral infection in vivo (15). More recently, it has been demonstrated that LXR in developing thymocytes reduces negative selection by limiting lipid rafts-mediated induction of Bim

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The authors declare no competing interest.

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(16). Moreover, mice with T cell-specific LXRβ deficiency harbor defective Treg cells and develop fatal autoimmune diseases (17). These studies collectively demonstrate an essential regulatory role of LXRs in T cell development, T cell activation upon antigen-recognition, and the function of regulatory T cells. However, the cell-intrinsic role of LXRs in diverse helper T cell subsets remains unclear.

In the present study, we aimed to investigate the role of LXRs on the differentiation and function of helper T cell subsets in vivo. While LXRβ-deficient T cells showed enhanced Th1 and Th17 differentiation in vitro, we did not observe any evident differences in the frequency of these helper T cell subsets between the wild-type (WT) and LXRβ-deficient $CD4^+$ T cell population in a mixed bone marrow (BM) chimera in vivo. Instead, we observed a significant increase in the frequency of Tfh cells in the LXRβ-deficient $CD4^+$ T cell population in the same mixed BM chimera, indicating a selective and cell-intrinsic regulation of Tfh cell differentiation by LXRβ. Mechanistic studies revealed that LXR inhibited the GSK3β-TCF1 signaling pathway in murine and human $CD4^+$ T cells. These findings identify LXRβ as a negative regulator of TCF-1 during the differentiation of Tfh cells.

Results

LXRβ Regulates the Development and Activation of $CD4^+$ T Cells. To investigate the role of LXRs in $CD4^+$ T cells, we first compared the levels of intracellular lipids in various $CD4^+$ T cells. BODIPY staining showed a higher level of intracellular lipids in $CD44^{hi}$ effector/memory $CD4^+$ T cells than naïve ones (*SI Appendix,* Fig. S1*A*). Among genes involved in cholesterol synthesis, levels of *Pmvk*, *Fdft1*, *Sqle*, *Nsdhl*, and *Dhcr7* were higher in the former (*SI Appendix,* Fig. S1*B*). Similarly, levels of *Sult2b1a* and *Cyp27a1*, genes involved in cholesterol metabolism, were significantly higher in the effector/memory $CD4^+$ T cells (*SI Appendix,* Fig. S1*C*). The levels of *Nr1h2* (encoding LXRβ) and *Rxrb* (encoding RXRβ) were higher than those of *Nr1h3* (encoding LXRα) and *Rxra* (encoding RXRα), respectively, in both naïve and effector/memory $CD4^+$ T cells (*SI Appendix,* Fig. S1*D*), suggesting that LXRβ and RXRβ are dominant isoforms in $CD4^+$ T cells. The levels of *Nr1h2* and *Rxrb* were diminished in effector/memory $CD4^+$ T cells. We next determined the kinetics of transcripts encoding enzymes involved in the cholesterol synthesis pathway in $CD4^+$ T cells after stimulation with anti-CD3 and anti-CD28. Consistent with a previous study (15), activation of $CD4^+$ T cells augmented the expression of genes related to cholesterol synthesis in a time-dependent manner with a concomitant increase in intracellular lipids (*SI Appendix,* Fig. S1 *E* and *F*). The levels of *Sult2b1a,* and *Cyp11a1* increased rapidly by 4 h after stimulation (*SI Appendix,* Fig. S1*G*), while those of *Nr1h3*, *Srebf2*, and *Rxrb* were all slightly decreased by 24 h (*SI Appendix,* Fig. S1*H*).

To determine the role of LXRβ in the T cell development, we analyzed thymocytes and found that $Nr1h2^{-/-}$ mice harbored a slightly elevated number of thymocytes and an increased frequency of $CD4^+CD8^+$ population with a concomitant decrease in the $CD4^+CD8^-$ and $CD4^-CD8^+$ population, all of which are consistent with a previous study (16) (*SI Appendix,* Fig. S2 *A* and *B*). The frequency of $Foxp3^+$ cells within the $CD4^+CD8^-$ population was also slightly reduced in the $Nr1h2^{-/-}$ mice (*SI Appendix,* Fig. S2*C*). $Nr1h2^{-/-}$ mice exhibited a decrease in the frequency of $CD4^+$ T cells in the secondary lymphoid organs, at least in part, due to an impaired thymic T cell development and output (16). In addition, we observed a general increment of the effector/memory population within $CD4^+$ T cells (*SI Appendix,* Fig. S2*D*). Likewise, activated $Nr1h2^{-/-}$ $CD4^+$ T cells via anti-CD3 and anti-CD28 displayed a more robust proliferation than those of WT, associated with an increased expression of IL-2 and CD25 (*SI Appendix,* Fig. S3 *A* and *B*). These observations demonstrate that diverse subsets of helper T cells as well as regulatory T cells are differentially regulated in the $Nr1h2^{-/-}$ mice at a steady state, and that LXRβ controls the TCR-mediated activation and division of $CD4^+$ T cells.

LXRβ Suppresses Tfh Cell Differentiation in a Cell-Intrinsic Manner. To address whether LXRβ impacts the differentiation of $CD4^+$ T cells in vitro, naïve $CD4^+$ T cells were cultured under Th1-, Th2-, Th17-, and Treg-skewing conditions, respectively. We found that the frequencies of Th1 and Th17 cells were higher in $Nr1h2^{-/-}$ T cells than in the WT ones, while those of Th2 and Treg cells were comparable (*SI Appendix,* Fig. S3 *C–F*). To investigate whether LXRβ impacts the differentiation of naïve $CD4^+$ T cells into effector subsets of helper T cells in vivo, we analyzed Peyer's patches where spontaneous germinal center (GC) reaction occurs in naive mice. Among $CD44^{hi}CD4^+$ T cells, the frequency of Th1 cells and Th17 cells was higher and lower, respectively, in the $Nr1h2^{-/-}$ mice compared with those in the WT mice (*SI Appendix,* Fig. S4*A*). Unlike the thymocytes, we observed comparable frequencies of $Foxp3^+$ Treg cells between the two groups (*SI Appendix,* Fig. S4*B*). Of note, the frequency of $PD\text{-}1^+CXCR5^+$ Tfh cells and $PD\text{-}1^{hi}CXCR5^{hi}$ GC Tfh were found to be significantly higher in Peyer's patches of the $Nr1h2^{-/-}$ mice (Fig. 1*A*).

Next, we compared Th subsets in the draining lymph nodes (LNs) between the WT and the $Nr1h2^{-/-}$ mice after immunization with keyhole limpet hemocyanin (KLH) emulsified in Freund's Adjuvant, Complete (CFA). Frequencies of Th1 and Th17 cells were comparable (*SI Appendix,* Fig. S4*C*); however, we observed a significant increase in $PD\text{-}1^+CXCR5^+Foxp3^-$ Tfh cells, particularly the $PD\text{-}1^{hi}CXCR5^{hi}$ GC Tfh population, in the $Nr1h2^{-/-}$ mice (Fig. 1*B*). We also observed a similar increase in the $PD\text{-}1^+CXCR5^+Foxp3^+$ Tfr cells (Fig. 1*C*). Upon immunization with NP-Ovalbumin (OVA) emulsified in CFA, $Nr1h2^{-/-}$ mice showed a significantly increased frequency of $GL7^+$ $B220^+$ GC B cells and had a slight but significant increase in the serum levels of high-affinity NP_7-specific IgG in comparison with WT mice, while the levels of global affinity NP_{29}-specific IgG were comparable between the two groups (*SI Appendix,* Fig. S4 *D* and *E*). To test if LXRβ also impacts Tfh cell responses generated upon viral infection, we employed the LCMV-Armstrong infection model. We observed a significant increase in Tfh cell among $CD4^+$ T population in the spleen of $Nr1h2^{-/-}$ mice (Fig. 1*D*). The frequency of Th1 cell was increased in the $Nr1h2^{-/-}$ mice, while that of Treg cell was comparable (*SI Appendix,* Fig. S4 *F* and *G*). To determine whether LXRα also impacts Tfh cell responses in vivo, we immunized the WT and $Nr1h3^{-/-}$ mice with KLH emulsified in CFA and observed a comparable Tfh cell population between the two groups, indicating that LXRβ, but not LXRα, represses Tfh cell responses in vivo (Fig. 1*E*).

To determine LXRβ's role in the Tfh cells' ability to promote B cells in vitro, we flow-sorted either the WT or $Nr1h2^{-/-}$ Tfh cells and cocultured them with WT naïve B cells in the presence of anti-IgM and anti-CD3 for 7 d. We observed comparable levels of IgG and IgG1 in the supernatant (*SI Appendix,* Fig. S4*H*). Thus, LXRβ-deficient Tfh cells were as efficient as WT Tfh cells in stimulating B cells to produce immunoglobulin.

To interrogate the cell-intrinsic role of LXRβ in helper T cell responses in vivo, we generated mixed BM chimera by transferring 1:1 mixture of WT (CD45.1) and $Nr1h2^{-/-}$ (CD45.2) BM cells into the BM-ablated $Rag1^{-/-}$ recipients. These recipients were

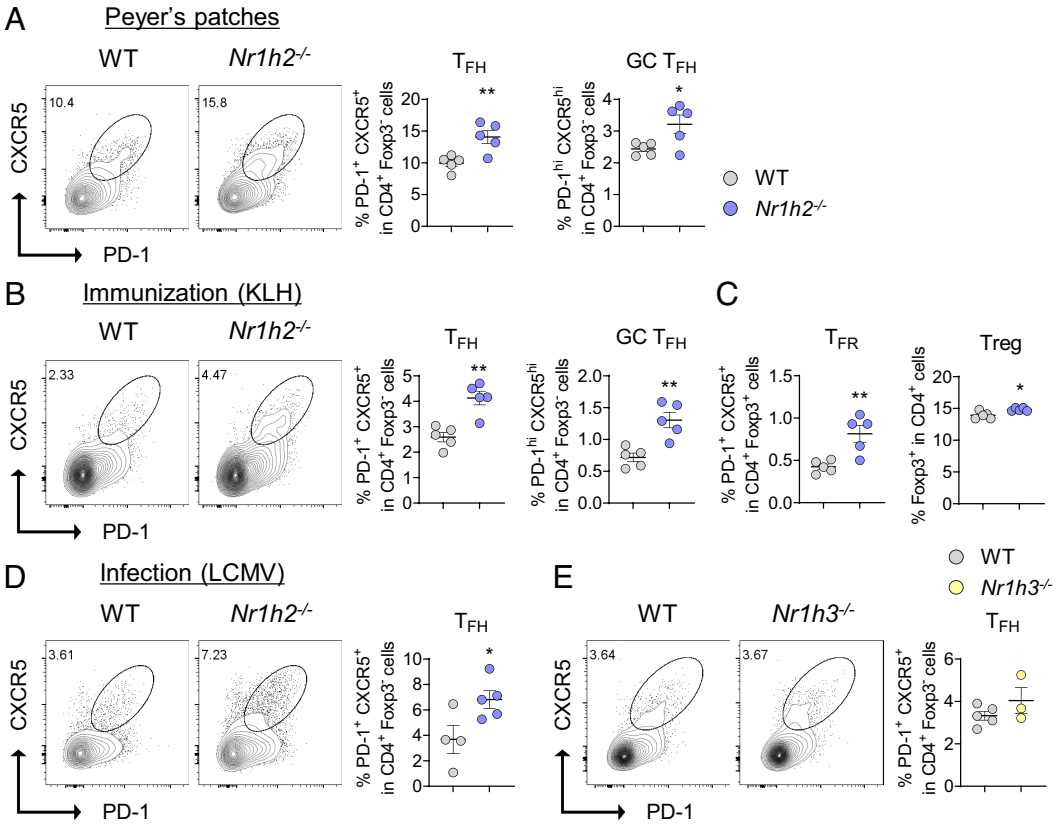

Fig. 1. LXRβ deficiency leads to enhanced Tfh cell differentiation in vivo. (*A*) Analysis of Tfh cell population in Peyer's patch at steady state. Representative Fluorescence-activated cell sorting (FACS) plots of PD-1$^+$CXCR5$^+$ total Tfh within CD4$^+$Foxp3$^-$ cells, and the frequencies of total Tfh and PD-1^{hi}CXCR5^{hi} GC Tfh cell frequencies (n = 5). (*B* and *C*) WT and *Nr1h2*$^{-/-}$ mice were immunized with KLH in CFA and draining LNs were analyzed on day 8 (n = 5). (*B*) Representative FACS plots and frequencies of Tfh (PD-1$^+$CXCR5$^+$) cells within CD4$^+$Foxp3$^-$ cells. Tfh and GC Tfh cell frequencies. (*C*) Tfr and Treg cell frequencies. (*D*) WT and *Nr1h2*$^{-/-}$ mice were infected with 2 × 10^5 plaque-forming units (pfu) of LCMV-Armstrong, and spleens were analyzed on day 8. Representative FACS plots and frequency of Tfh cells (n = 4 to 5). (*E*) WT and *Nr1h3*$^{-/-}$ mice were immunized with KLH in CFA and draining LNs were analyzed on day 8 (n = 3 to 5). Representative FACS plots of Tfh cells and frequencies. Data are representative of at least two independent experiments. Quantification plots show the mean ± SEM; *P < 0.05 and **P < 0.01.

subsequently immunized with KLH emulsified in CFA, and Th subsets in the draining LNs were analyzed (Fig. 2*A*). The frequencies of Th1, Th17, and Treg cells were indistinguishable between the WT and *Nr1h2*$^{-/-}$ compartments within the chimeras (Fig. 2*B*). By contrast, the frequencies of Tfh, GC Tfh, and Tfr cells were all significantly higher in the *Nr1h2*$^{-/-}$ compartments of the same recipients (Fig. 2*C*). Furthermore, we observed a significant increase in Tfh cells in the *Nr1h2*$^{-/-}$ compartment within the mixed BM chimeras infected with LCMV-Armstrong, indicating a cell-intrinsic inhibitory role of LXRβ in the differentiation of Tfh cells in vivo (Fig. 2*D*). Unlike that of Tfh cells, the frequency of GC B cells was indistinguishable between the WT and *Nr1h2*$^{-/-}$ compartment (*SI Appendix*, Fig. S4*I*). To further confirm cell-intrinsic regulation of LXRβ in antigen-specific CD4$^+$ T cell responses in vivo, we cotransferred WT OT-II (CD45.1$^+$CD45.2$^+$) and *Nr1h2*$^{-/-}$ OT-II T cells (CD45.2$^+$) into CD45.1$^+$congenic mice before immunizing the recipients with OVA emulsified in CFA (Fig. 2*E*). Consistent with the observations in mixed BM chimeras, we observed a significant increase in Tfh cells within the *Nr1h2*$^{-/-}$ OT-II T cell population in comparison with the WT counterpart (Fig. 2*F*). Together, these findings demonstrate that LXRβ negatively regulates Tfh cell differentiation in vivo in a cell-intrinsic manner.

LXRβ Represses GSK3β Phosphorylation to Down-Regulate TCF-1 in CD4$^+$ T Cells. We next sought to dissect the mechanism through which LXRβ suppresses Tfh cell differentiation. CXCR5, PD-1, Bcl6, and TCF-1 represent Tfh cell signature molecules. When we compared the expression of these molecules in the mixed BM chimeras described in Fig. 2*A*, the levels of CXCR5, PD-1, and Bcl6 did not differ between WT and *Nr1h2*$^{-/-}$ Tfh cells (Fig. 3 *A* and *B*). By contrast, the expression of TCF-1 was higher in *Nr1h2*$^{-/-}$ Tfh cells than that in the WT (Fig. 3*B*). *Il21* level in the WT Tfh cells and *Nr1h2*$^{-/-}$ Tfh cells was tantamount (Fig. 3*C*). Consistently, we also observed higher TCF-1 in *Nr1h2*$^{-/-}$ OT-II Tfh cells than WT OT-II Tfh cells in a cotransfer study, as depicted in Fig. 3 *D* and *E*. Unlike the mixed BM chimera study, the level of PD-1 was slightly lower in *Nr1h2*$^{-/-}$ OT-II Tfh cells. These findings prompted us to hypothesize that LXRβ inhibits Tfh cell differentiation via TCF-1 repression.

To probe the mechanistic details by which LXRβ controls TCF-1 in Tfh cells in vivo in an unbiased way, we subjected flow-sorted Tfh cells isolated from LCMV-infected *Nr1h2*$^{-/-}$ mice and WT mice to RNA-sequencing analysis. At a setting of fold change >1.5, and false discovery rate <0.1, 101 genes were up-regulated, and 34 genes were down-regulated in the LXRβ-deficient Tfh cells. As expected, *Nr1h2* and well-known LXR target genes, *Ldlr*, *Mylip,* and *Abcg1* were down-regulated in *Nr1h2*$^{-/-}$ Tfh cells. Of note, we found that among the up-regulated differentially expressed genes (DEGs) were genes previously known to be positively associated with the Tfh cell lineage program, such as *Spp1, C3, Vdr*, and *Fosl*, and among the down-regulated DEGs were genes known to be negatively associated with the Tfh cell lineage program, such as *Vamp7*, and *Dock7* (*SI Appendix,* Fig. S5 *A* and *B*) (18, 19). In addition, gene set enrichment analysis (GSEA)

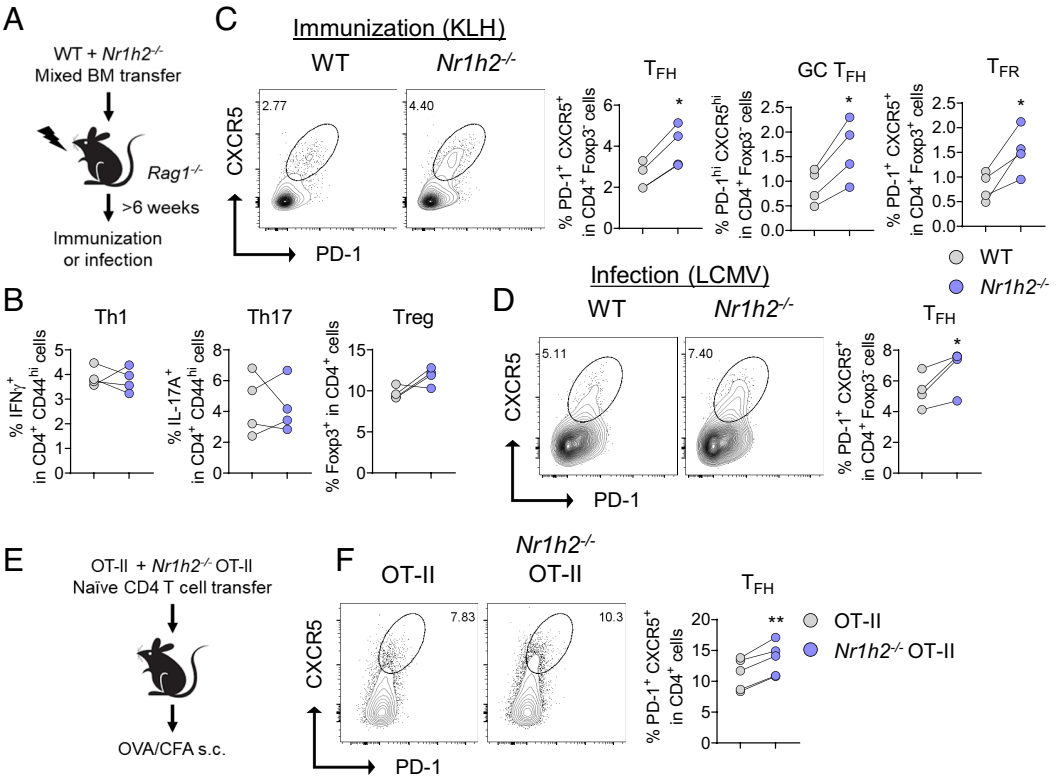

Fig. 2. LXRβ inhibits Tfh cell differentiation in a cell-intrinsic manner. (*A–D*) BM chimeric mice were generated by adoptive transfer of 1:1 mixture of WT and *Nr1h2*$^{-/-}$ BM into irradiated *Rag1*$^{-/-}$ mice. After the reconstitution period, the chimeras were either s.c. immunized with KLH in CFA (*B* and *C*) or infected with LCMV (*D*) before flow cytometric analysis (n = 4). (*A*) Scheme of the mixed BM chimera experiments. (*B*) Th1, Th17, and Treg cell frequencies. (*C*) Representative FACS plots of Tfh cells, and frequencies of Tfh, GC Tfh, and Tfr cells in the draining LNs. (*D*) Representative FACS plots and frequency of Tfh cells. (*E* and *F*) B6.SJL (CD45.1^{+}) mice were i.v. transferred with mixture of WT (CD45.1/2^{+}) and *Nr1h2*$^{-/-}$ (CD45.2^{+}) OT-II T cells. The recipients were immunized with OVA in CFA and the draining LNs were analyzed on day 8 (n = 5). (*E*) Schematic representation of the OT-II cotransfer experiments. (*F*) Representative FACS plots and frequency of Tfh cells. Data are representative of at least two independent experiments. Quantification plots show the mean ± SEM; *$P < 0.05$, and **$P < 0.01$.

revealed that *Nr1h2*$^{-/-}$ Tfh cells were enriched for genes involved in the BCL6-high Tfh program, indicating that GC Tfh transcriptome is up-regulated in the absence of LXRβ (*SI Appendix,* Fig. S5*C*). In addition, ERK pathway as well as PI3K-AKT signaling pathway was enriched in *Nr1h2*$^{-/-}$ Tfh cells (Fig. 4 *A* and *B* and *SI Appendix,* Fig. S5*D*). Indeed, when stimulated with anti-CD3 and anti-CD28, *Nr1h2*$^{-/-}$ CD4^{+} T cells showed higher levels of phosphorylated forms of ERK and AKT than WT CD4^{+} T cells (Fig. 4 *D* and *E*). Conversely, addition of an LXR agonist, GW3965, significantly suppressed the phosphorylation of ERK as well as AKT in CD4^{+} T cells (Fig. 4 *F* and *G*). Since AKT induces the activation of mTORC1 pathway, we tested whether downstream molecules of mTORC1 are regulated by LXRβ in CD4^{+} T cells. We observed an enhanced level of phospho-S6 in the *Nr1h2*$^{-/-}$ CD4^{+} T cells, and a delayed phosphorylation of S6 by GW3965 treatment (*SI Appendix,* Fig. S5 *E* and *F*). Thus, LXRβ controls the activation of AKT-mTORC1 and ERK in CD4^{+} T cells after TCR stimulation.

As illustrated in Fig. 4*C*, activation of ERK, PI3K/AKT, and Wnt commonly induces the phosphorylation of GSK3β, leading to the release and translocation of β-catenin to the nucleus. TCF-1 contains β-catenin-binding domain, and TCF-1/β-catenin complex facilitates early differentiation of Tfh cell by inhibiting *Prdm1* (encoding Blimp1) and *Il2ra,* and by antagonizing Bcl6-mediated autoinhibitory effect in developing Tfh cells (20, 21). Moreover, inhibition of GSK3β kinase activity increases stability and nuclear translocation of β-catenin and up-regulates TCF-1 (22, 23). Thus, we hypothesized that LXRβ represses TCF-1 expression in helper T cells by regulating GSK3β. Indeed, the level of phospho-GSK3β was significantly increased in the *Nr1h2*$^{-/-}$ CD4^{+} T cells but was remarkably diminished by GW3965 treatment in the WT CD4^{+} T cells (Fig. 4 *H* and *I*). Thus, LXRβ negatively regulates the phosphorylation of GSK3β in CD4^{+} T cells.

Naïve and recently activated CD4^{+} T cells maintain high levels of TCF-1; however, activated T cells down-regulate TCF-1 after four to five divisions (24). To determine whether LXRβ regulates TCR-mediated downregulation of TCF-1, we measured TCF-1 levels after stimulating naïve CD4^{+} T cells with anti-CD3 and anti-CD28 and found a significantly higher level of TCF-1 in *Nr1h2*$^{-/-}$ T cells than that in WT T cells (Fig. 4*J*). Conversely, the addition of an LXR agonist significantly decreased TCF-1 in activated CD4^{+} T cells (Fig. 4*K*). Despite the observed differences in the level of TCF-1, we found no evident difference in the transcript levels of *Tcf7* (encoding TCF-1) between WT and *Nr1h2*$^{-/-}$ T cells, nor between vehicle- and GW3965-treated CD4^{+} T cells (*SI Appendix*, Fig. S5 *G–I*), suggesting a posttranscriptional/translational regulation of TCF-1 by LXR. TCF-1 and LEF-1 contain a conserved high-mobility-group DNA-binding domain, and coordinate Tfh cell differentiation (20). Unlike TCF-1, however, the levels of LEF-1 were comparable between WT and *Nr1h2*$^{-/-}$ CD4^{+} T cells as well as between vehicle- and GW3965-treated CD4^{+} T cells (*SI Appendix*, Fig. S5 *J* and *K*).

We next asked whether LXRβ-mediated downregulation of phospho-GSK3β accounts for the increased TCF-1 observed in LXRβ-deficient T cells. As expected, treatment with a Wnt/β-catenin agonist BIO increased TCF-1 expression in WT CD4^{+} T cells. The addition of an LXR agonist, however, almost completely abolished the upregulation of TCF-1 induced by BIO (*SI Appendix,* Fig. S5*L*). To investigate whether LXR would also regulate TCF-1

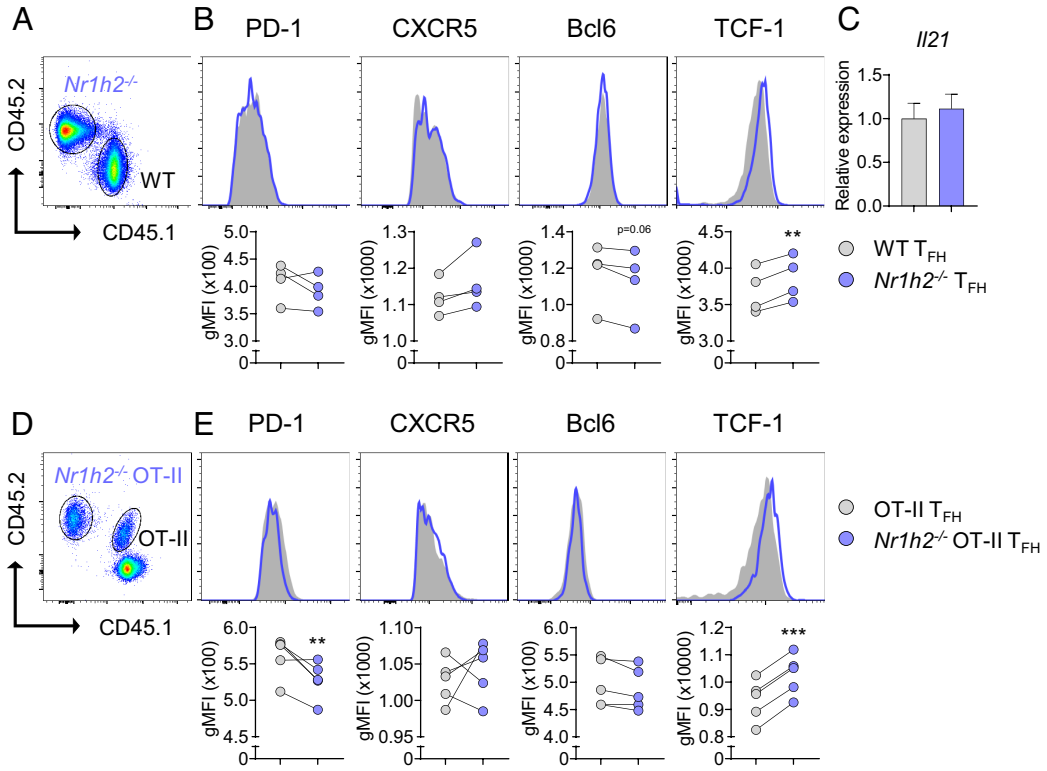

Fig. 3. LXRβ negatively regulates TCF-1 in Tfh cells. (*A* and *B*) BM chimera mice were generated and immunized with KLH in CFA as in Fig. 2*A* (n = 4). (*A*) A representative FACS plot of CD45.1$^+$ WT cells and CD45.2$^+$ *Nr1h2*$^{-/-}$ cells in the draining LNs. (*B*) Geometric mean fluorescence intensities (gMFIs) of PD-1, CXCR5, Bcl6, and TCF-1 in gated Tfh cells in WT and *Nr1h2*$^{-/-}$ Tfh cells. (*C*) Tfh cells from WT and *Nr1h2*$^{-/-}$ mice were flow-sorted and the relative expression of *Il21* was analyzed by qRT-PCR (n = 4). (*D* and *E*) B6.SJL (CD45.1$^+$) mice were i.v. transferred with mixture of WT (CD45.1/2$^+$) and *Nr1h2*$^{-/-}$ (CD45.2$^+$) OT-II T cells, followed by s.c. immunization with OVA in CFA and subsequent flow cytometric analysis in the draining LNs (n = 5). (*D*) Representative FACS plot of CD45.1$^+$CD45.2$^+$ WT and CD45.2$^+$ *Nr1h2*$^{-/-}$ OT-II T cells. (*E*) gMFIs of PD-1, CXCR5, Bcl6, and TCF-1 in gated Tfh cells in the indicated Tfh cells (n = 5). Data are representative of three independent experiments. Quantification plots show the mean ± SEM; $^{**}P < 0.01$, and $^{***}P < 0.001$.

independently of TCR stimulation, naïve CD4$^+$ T cells were treated with BIO without TCR stimulation. Likewise, GW3965 canceled TCF-1 upregulation induced by BIO (Fig. 4*L*). The same trend was observed when BIO and GW3965 were treated 1 d after TCR stimulation or under IL-2 stimulation conditions (*SI Appendix*, Fig. S5 *M* and *N*). We next addressed if the observed GW3965-mediated downregulation of TCF-1 was mainly due to antiproliferative effect of the agonist, and found that GW3965 significantly down-regulated TCF-1 expression in activated CD4$^+$ T cells regardless of the division (*SI Appendix*, Fig. S5*O*). Collectively, these findings indicate that LXR inhibits not only TCR-induced activation of PI3K/AKT and ERK but also Wnt/β-catenin signaling to down-regulate TCF-1 expression in CD4$^+$ T cells.

LXR activation is known to promote cholesterol efflux by up-regulating the ATP-binding cassette (ABC) transporters ABCA1 and ABCG1 (25). Accordingly, GW3965 treatment not only increased the expression of *Abca1* and *Abcg1* mRNA but also decreased the levels of intracellular cholesterol in CD4$^+$ T cells (*SI Appendix*, Fig. S5 *P* and *Q*). Since LXR activation down-regulates intracellular sterol level, we questioned whether the inhibitory effect of LXR can be restored by mevalonate, a precursor for cholesterol and oxysterols (15). Of note, higher concentration of mevalonate (5 mM) almost completely abolished the GW3965-induced downregulation of TCF-1, while lower concentration of mevalonate (100 μM) failed to do so (Fig. 4*M*). Given that the lower concentration of mevalonate is known to be sufficient for nonsteroidal modifications while insufficient to drive sterol synthesis (15), this result suggests that LXR inhibits TCF-1 expression, at least in part, by lowering intracellular sterol in CD4$^+$ T cells.

Activation of LXR Attenuates GC Reactions and Antigen-Specific IgG Production. The observed regulatory role of LXRβ on TCF-1 expression and Tfh cell differentiation proposed that LXR agonism could attenuate GC reactions and subsequent immunoglobulin production in vivo. To address this point, we again employed a mouse model of immunization with KLH emulsified in CFA, and analyzed Tfh, GL7$^+$B220$^+$ GC B cells, and CD138$^+$B220$^-$ plasma cells in the draining LNs as well as the levels of KLH-specific Igs in the serum (Fig. 5*A*). As depicted in Fig. 5 *B*–*D*, we observed a significant reduction in the frequencies and numbers of Tfh cells, GC B cells, and plasma cells in the draining LNs of GW3965-treated mice compared with vehicle-treated control mice. As a consequence, the serum levels of KLH-specific IgG, particularly IgG2c, were also significantly reduced in the former, while that of IgM remained comparable between the two groups (Fig. 5*E*). To study the definitive function of T cell-specific LXR activation, we stimulated naïve OT-II T cells under Tfh-like skewing condition in the presence of GW3965 or vehicle and transferred them into congenic mice before immunizing the recipients with NP-OVA emulsified in CFA (*SI Appendix*, Fig. S6*A*) (18). CTV-dilution assay showed that pretreatment of GW3965 during the Tfh-like cell differentiation in vitro did not affect the antigen-driven proliferative potential of the resultant OT-II T cells (*SI Appendix*, Fig. S6*B*). GW3965-treated OT-II cells were defective in their ability to become Tfh cells in vivo and were much less effective in inducing GC B cells, and early plasmablasts (*SI Appendix*, Fig. S6 *C* and *D*). Consequently, the levels of NP_7-specific IgG in the serum, particularly IgG2b and IgG2c, were also significantly diminished in the recipients of GW3965-treated Tfh-like cells (*SI Appendix*,

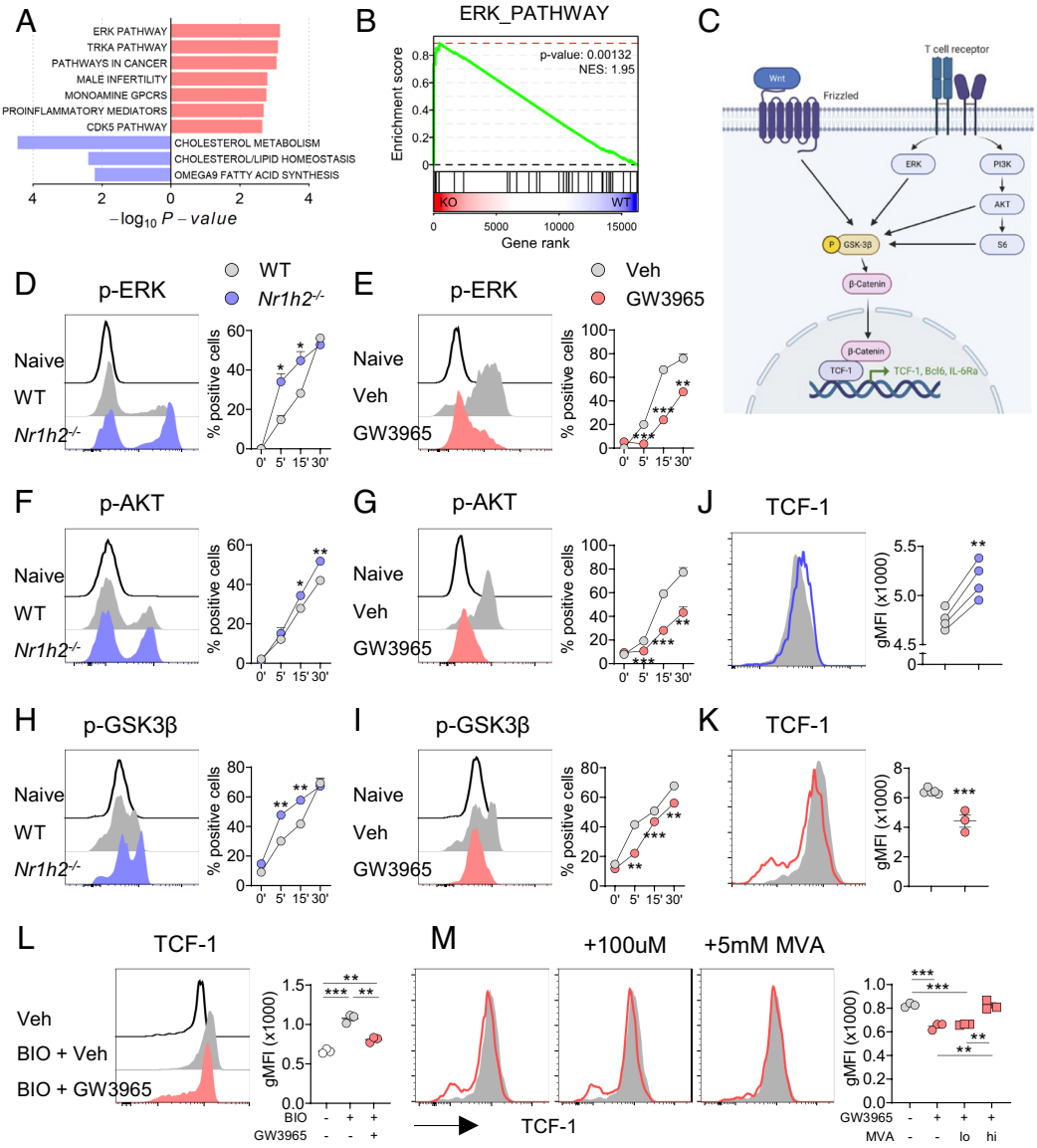

Fig. 4. LXR inhibits PI3K/AKT, ERK, and Wnt/β-catenin signaling to repress TCF-1 in CD4$^+$ T cells. (*A* and *B*) Tfh cells from the spleens of LCMV-Armstrong-infected WT and *Nr1h2*$^{-/-}$ mice were subjected to bulk RNA-seq analysis. (*A*) Top enriched or down-regulated pathways. (*B*) GSEA of ERK_PATHWAY (*C*) Schematic view of TCR and Wnt signaling pathway in T cells. (*D–K*) WT or *Nr1h2*$^{-/-}$ naïve CD4$^+$ T cells were stimulated with anti-CD3 and anti-CD28 in the presence of vehicle or GW3965, and the levels of indicated molecules were determined at each time point (n = 3 to 4). (*D* and *F*) Representative histograms of p-ERK expression, and frequencies of p-ERK$^+$ cells. (*E* and *G*) Representative histograms of p-AKT expression, and frequencies of p-AKT$^+$ cells. (*H* and *I*) Representative histograms of p-GSK3β expression, and frequencies of p-GSK3β$^+$ cells. (*J* and *K*) WT or *Nr1h2*$^{-/-}$ naïve CD4$^+$ T cells were stimulated with anti-CD3 and anti-CD28 in the presence of vehicle or GW3965 and analyzed for the expression of TCF-1 (n = 3 to 5). (*L*) Naïve CD4$^+$ T cells were cultured with BIO in the presence of vehicle or GW3965 and gMFIs of TCF-1 were examined (n = 3). (*M*) Naïve CD4$^+$ T cells were cultured with GW3965 in the presence of vehicle or mevalonate and gMFIs of TCF-1 were examined (n = 4). Data are representative of three independent experiments. Quantification plots show the mean ± SEM; *P < 0.05, **P < 0.01, and ***P < 0.001.

Fig. S6*E*). Thus, treatment with an LXR agonist significantly dampened Tfh cell differentiation from naïve precursors, which led to diminished GC reactions and subsequent T cell-dependent IgG production in vivo.

LXR Controls the Differentiation of Human Tfh Cell and TCF-1 Expression. Finally, we sought to determine the effects of LXR agonism on the differentiation of human Tfh cells. To this end, we labeled flow-sorted naïve CD4$^+$ T cells from healthy donors with vital dye CFSE before stimulating them under Tfh-skewing condition in the presence of IL-12 and TGF-β (Fig. 6*A*) (26, 27). Consistent with the data observed in multiple murine models, treatment with the LXR agonist significantly diminished the frequencies of BCL6$^+$CXCR5$^+$ and ICOS$^+$CXCR5$^+$ cells (Fig. 6 *B* and *C*). CFSE-dilution analysis showed that GW3965 treatment induced a similar significant reduction in the frequency of BCL6$^+$CXCR5$^+$ population in both undivided and 3 to 4 times divided CD4$^+$ T cells in this experimental setting (*SI Appendix*, Fig. S7 *A*). To determine whether LXR also controls TCF-1 expression in human CD4$^+$ T cells, we stimulated naïve CD4$^+$ T cells with anti-CD3 and anti-CD28 and found that GW3965 treatment remarkably down-regulated TCF-1 levels (Fig. 6*D*). Consistently, the levels of TCF-1 expression were all significantly diminished by GW3965 regardless of the degree of T cell division (*SI Appendix*, Fig. S7 *B*). Moreover, while TCF-1 expression was increased by BIO, GW3965 almost completely abolished the upregulation of TCF-1 induced by BIO in human CD4$^+$ T cells (Fig. 6*E*). Together with the results obtained in the murine

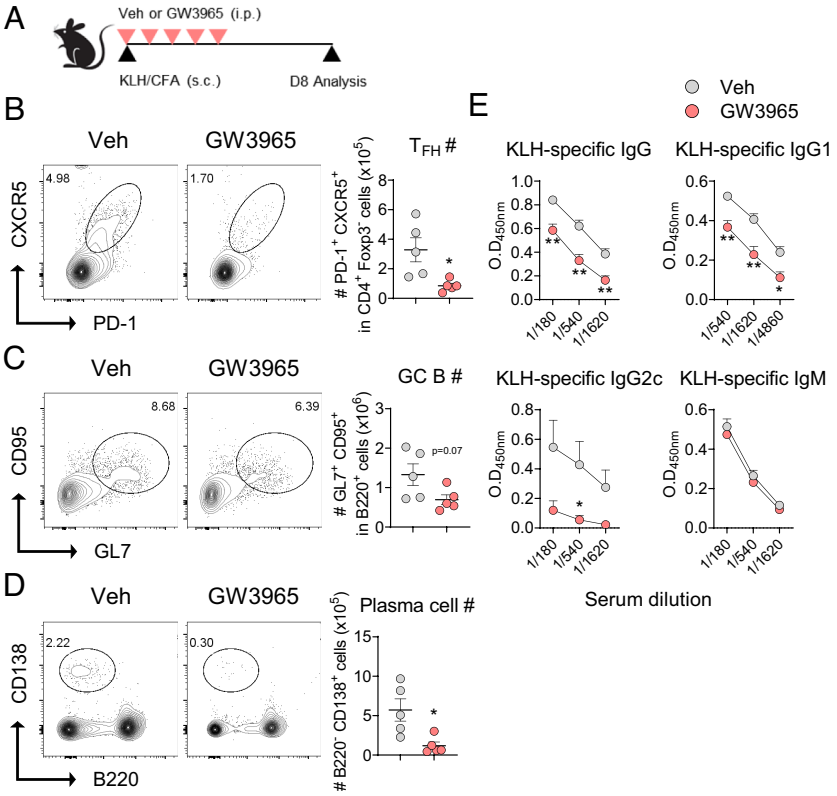

Fig. 5. LXR activation diminishes GC response in vivo. (*A–D*) Groups of mice were s.c. immunized with KLH emulsified in CFA and i.p. injected with vehicle or GW3965. Sera and lymphoid cells from the draining LNs were analyzed at day 8 (n = 5). (*A*) Scheme of the experiment. Representative FACS plots and absolute numbers (#) of Tfh cells (*B*), GC B cells (*C*), and plasma cells (*D*). (*E*) Levels of KLH-specific IgG, IgG1, IgG2c, and IgM in the serum. Data are representative of two independent experiments. Quantification plots show the mean ± SEM; $*P < 0.05$, and $**P < 0.01$.

models, these results strongly suggest that LXR inhibits the differentiation of Tfh cells by repressing the expression of TCF-1 in human $CD4^+$ T cells.

Discussion

In the present study, we investigated the cell-intrinsic role of LXRβ in helper T cell subsets in vivo. Our findings unveil a crucial regulatory role of LXRβ in the Tfh cell lineage program by demonstrating that i) the frequency of Tfh cells, but not the other subsets of helper T cells, was increased in the LXRβ-deficient population in the mixed BM chimera and OT-II cotransfer studies, ii) LXRβ-deficient Tfh cells expressed higher levels of TCF-1 than WT Tfh cells, iii) activation of LXR down-regulated TCF-1 expression in murine and human $CD4^+$ T cells, iv) treatment with an LXR agonist suppressed GC reactions and high-affinity IgG production in response to protein immunization, and v) LXR activation inhibited human Tfh cell differentiation regardless of the degree of T cell division. Based on these findings, we propose that LXRβ is a cell-autonomous negative regulator of Tfh cell differentiation through the repression of TCF-1.

Several studies have demonstrated a regulatory role of LXR in humoral immunity and antibody-mediated autoimmunity. For instance, LXRαβ-deficient mice spontaneously develop lupus-like phenotypes by the age of 10 mo, and treatment with an LXR agonist ameliorates lupus symptoms in lupus-prone mice (14). Defective clearance of apoptotic cells has been proposed as a cause of lupus-like phenotype in the LXRαβ-deficient mice. Similarly, treatment with LXR agonists induces preventive and therapeutic effects in animal models of collagen-induced arthritis (28, 29). Our recent study also showed that downregulation of LXR in dendritic cells leads to enhanced Tfh cell differentiation and subsequent humoral immunity in atherogenic mice (30). These studies have proposed that LXR negatively regulates humoral immunity by modulating the function of macrophages and dendritic cells. In addition to these immunoregulatory roles of LXR in innate immune cells, the present study revealed a T cell-intrinsic role of LXRβ in controlling Tfh cell differentiation by employing mixed BM chimeras and OT-II T cell cotransfer studies. The present study also proposes an additional cellular mechanism as uncontrolled autoreactive Tfh cell differentiation might contribute to autoantibody production in the LXRαβ-deficient mice. Thus, LXR acts as a critical checkpoint in macrophages, dendritic cells, and T cells to prevent unnecessary humoral immune responses.

Among Tfh cell signature molecules, LXRβ-deficient $CD4^+$ T cells expressed increased levels of TCF-1 in multiple in vivo and in vitro experimental settings. How TCF-1 is regulated in T cells has been obscure. $CD4^+$ T cells are known to down-regulate TCF-1 after a few divisions upon TCR stimulation; however, LXRβ-deficient $CD4^+$ T cells expressed higher TCF-1 despite their enhanced proliferative potential, suggesting that LXRβ actively represses TCF-1 in activated T cells. Supporting this notion, we observed that GW3965 suppressed TCF-1 expression regardless of the degree division in murine and human $CD4^+$ T cells. TCF-1 was initially discovered as a critical regulator of T cell development in the thymus and has been actively studied as an essential factor required for the stemness and memory response in T cells (31). More recent studies uncovered that TCF-1, together with LEF-1, plays a crucial role during the early differentiation of Tfh cells and Tfr cells by upregulating *Bcl6, Il6ra, Il6st*, and *Icos* (20, 32–35). Unlike TCF-1, LXR appeared to have little role in regulating LEF-1 expression in $CD4^+$ T cells. TCF-1-binding factors include

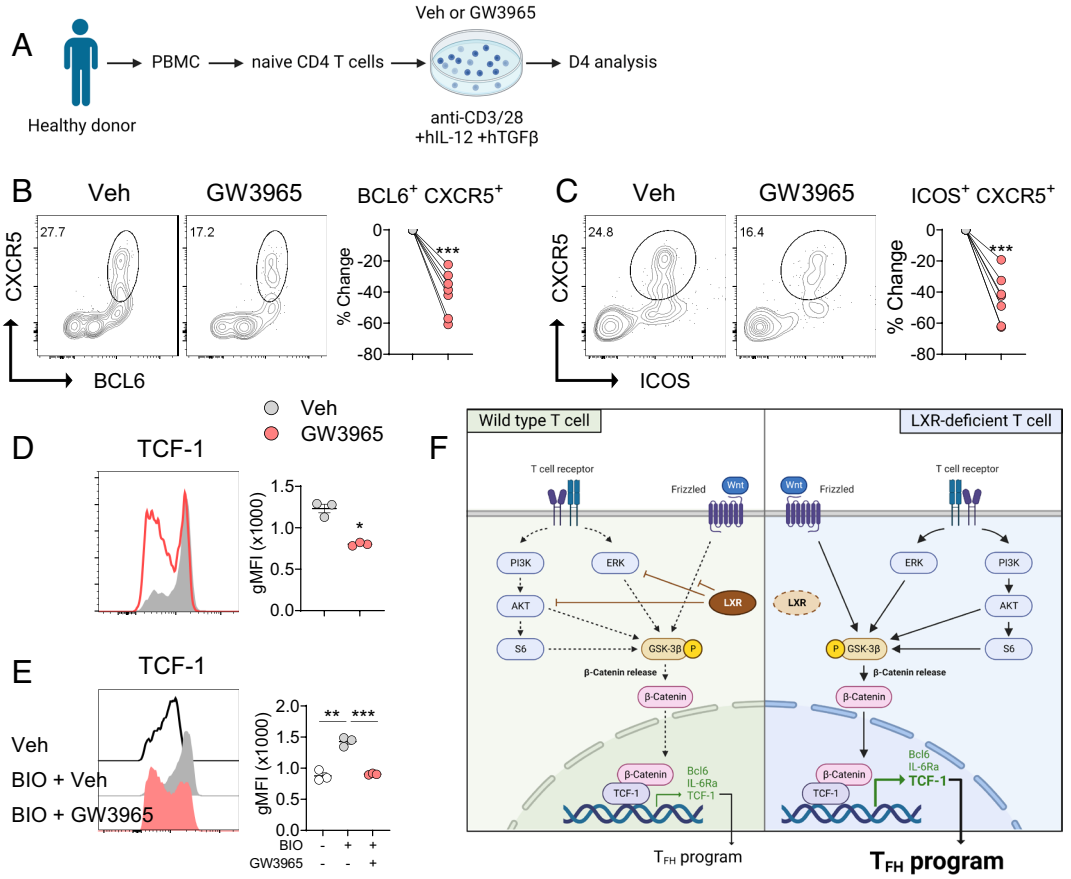

Fig. 6. LXR inhibits TCF-1 expression and Tfh cell differentiation in human CD4$^+$ T cells. (*A–C*) Flow-sorted human naive CD4$^+$ T cells were cultured under Tfh-skewing conditions before being analyzed by flow cytometry (n = 7). (*A*) Scheme of the experiment. (*B* and *C*) Percentage changes in BCL6$^+$CXCR5$^+$ cells (*B*) or ICOS$^+$CXCR5$^+$ cells (*C*) by GW3965 treatment. Each symbol represents an individual donor. (*D* and *E*) Human naive CD4$^+$ T cells were stimulated with anti-CD3 and anti-CD28 (*D*) or with BIO (*E*) in the presence of vehicle or GW3965 and analyzed for the expression of TCF-1 (n = 3). (*F*) Working model on the regulatory role of LXR during Tfh cell differentiation. Data are representative of three independent experiments. Quantification plots show the mean ± SEM; **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

β-catenin, Bcl6, EZH2, and Foxp3. The transcriptional activity of TCF-1 is regulated by β-catenin, which can be released after the phosphorylation of GSK3β (36). Phosphorylation of GSK3β is triggered by PI3K/AKT and ERK pathway downstream of TCR signaling as well as by Wnt signaling. Our present study demonstrates that LXR controls TCF-1 expression via two different mechanisms. First, LXR suppresses phosphorylation of GSK3β by inhibiting PI3K/AKT and ERK activation in activated CD4$^+$ T cells. This mechanism is supported by the enriched genes associated with PI3K-AKT signaling pathway in *Nr1h2*$^{-/-}$ Tfh cells in our GSEA analysis as well as increased phospho-AKT and phospho-ERK in *Nr1h2*$^{-/-}$ CD4$^+$ T cells upon TCR stimulation. Second, LXR suppresses Wnt/β-catenin pathway in a TCR-independent manner, since we observed that LXR activation almost completely abolished the upregulation of TCF-1 induced by a GSK3β inhibitor in resting CD4$^+$ T cells. These two mechanisms likely cooperate in CD4$^+$ T cells to fine-tune the activity of TCF-1 by regulating the translocation of β-catenin (Fig. 6*F*). Two isoforms of TCF-1 exist; unlike the long isoform, the short form of TCF-1 does not have a β-catenin-binding domain but has a binding domain for Bcl6 and Foxp3. Thus, LXR may control TCF-1 independently of β-catenin, which will be an interesting subject for future studies. The present study also suggests that LXR regulates TCF-1 expression through posttranscriptional/translational modification in T cells. There are several known mechanisms for posttranslational modification of TCF-1 (37). For instance, NLK and NLK-associated ring finger protein regulates the phosphorylation and ubiquitination of TCF-1 in human embryonic kidney cells (38, 39). Moreover, CK-1 has been shown to phosphorylate TCF-1 in mouse myoblast cells (40). Further studies will clarify the detailed mechanism by which LXR represses the expression of TCF-1 in CD4$^+$ T cells. In addition, TCF-1 is known to be required for maintaining stemness of T cells and regulating exhaustion, particularly in CD8$^+$ T cells (41). It would be interesting to investigate whether LXR is involved in the stemness and exhaustion of CD8$^+$ T cells via controlling TCF-1.

Previous studies showed that LXR activation has been shown to inhibit Th17 cell responses and suppress experimental autoimmune encephalomyelitis (EAE) in vivo (42, 43). While we also observed that *Nr1h2*$^{-/-}$ T cells showed enhanced Th17 cell differentiation in vitro, our immunization and infection models showed little difference in Th17 and Th1 cells between WT and *Nr1h2*$^{-/-}$ T cell compartments in mixed BM chimera studies, suggesting that the inhibitory role of LXR in Th17 cell responses could be through modulating innate immune cells including dendritic cells. LXR has been recently reported to prevent negative selection of developing thymocytes by reducing lipid rafts (16). Moreover, T cell-specific LXRβ-deficient mice exhibit impaired Treg cell functionality (17). This study also showed that Treg-specific deletion of LXRβ, even loss of a single copy of *Nr1h2* (*Foxp3*^{cre}*Nr1h2*$^{fl/+}$), results in fatal autoimmunity by 4 wk of age, similar to that observed in Scurfy mice. We observed a slight

decrease in the thymic Treg cells, but a normal frequency of Treg cells in the secondary lymphoid organs in *Nr1h2*$^{-/-}$ mice. We also did not observe any fatal autoimmune symptoms in *Nr1h2*$^{-/-}$ mice by 6 mo of age. Moreover, our mixed BM chimera study revealed no difference in the frequency of Foxp3^{+} Treg cells between the WT and *Nr1h2*$^{-/-}$ T cell compartments in the periphery, except for a slight increase in the CXCR5^{+}Foxp3^{+} Tfr cells in *Nr1h2*$^{-/-}$ mice. These findings strongly suggest that LXRβ is dispensable in Treg cell homeostasis, but suppresses differentiation of Tfr cells in a TCF-1–dependent manner (44). Similar to the present study, LXRαβ-deficient mice showed no defect in Foxp3^{+} Treg cells (14). Further studies are needed to illuminate the precise role of LXR in regulating the development and function of Foxp3^{+} Treg cells.

In summary, the present study outlines a previously unrecognized role for LXRβ in controlling Tfh cell differentiation via repression of TCF-1. Development of LXR synthetic agonists with selective induction of cholesterol efflux without inducing hypertriglyceridemia is an attractive area of therapeutic intervention, particularly for metabolic diseases. Our findings suggest that LXR could be also an attractive target for the treatment of autoimmune diseases caused by aberrant Tfh cell responses such as Pemphigus vulgaris, Sjogren's syndrome, and systemic lupus erythematosus (45). Conversely, since LXR activation can hamper humoral immunity against infections, LXR agonists in clinical settings should be given with caution to prevent the risk of infection.

Materials and Methods

A full description of the *Materials and Methods* is available in *SI Appendix*.

Ethics Statement. All animal experiments were performed according to protocols approved by Institutional Animal Care and Use Committees of Seoul National University (SNU 211025-7-2, SNU 200831-9-3, SNU 191210-3-11). Collection of human blood samples from healthy volunteers and subsequent experimental procedures were reviewed and approved by the Seoul National University Institutional Review Board (approval number: 2203/003-003), and all participants provided informed consent.

Animal Models. For immunization studies, mice were subcutaneously immunized with 50 μg KLH (Sigma-Aldrich) or 100 μg NP-OVA (Biosearch Technologies) emulsified in CFA (Sigma-Aldrich), and lymphoid cells from the draining LNs were stained and analyzed by flow cytometry on day 8 to 10. For viral infection experiments, mice were intraperitoneally injected with LCMV-Armstrong (2 × 10^{5} pfu), and lymphoid cells in the spleen were analyzed on day 8. In some experiments, mice were intraperitoneally treated with either Dimethyl sulfoxide (DMSO) vehicle or 20 mg/kg GW3965 (Tocris).

For BM chimera studies, 8- to 10-wk-old sublethally-irradiated *Rag1*$^{-/-}$ mice (9 Gy; X-RAD IR160, Precision X-Ray, USA) were i.v. injected 1:1 mixture of WT and *Nr1h2*$^{-/-}$ BM cells before 6 wk of reconstitution. The recipients were s.c. immunized with KLH in CFA or infected with LCMV, and lymphoid cells from the draining LNs or spleen were analyzed as indicated (46).

For OT-II T cell cotransfer studies, 1:1 mixture of flow-sorted WT and *Nr1h2*$^{-/-}$ naïve OT-II T cells (2 × 10^{6} cells) were i.v. transferred into sex-matched B6.SJL congenic recipient mice. One day later, the recipients were s.c. injected with 100 μg OVA (Sigma-Aldrich) emulsified in CFA and lymphoid cells from the draining LNs were analyzed on day 8.

CD4^{+} T Cell Stimulation In Vitro. CD4^{+} T cells from the spleen and peripheral LNs were positively selected with CD4 microbeads (L3T4; Miltenyi Biotec), and naive CD4^{+} T cells were further sorted as CD4^{+}CD25^{-}CD62L^{high}CD44^{low} cells with the FACSAria III cell sorter (Becton, Dickinson and Company (BD) Biosciences) and stimulated with plate-coated anti-CD3 (1 μg/mL, 145-2C11: BioXCell) and soluble anti-CD28 (1 μg/mL, 37.51; BioXCell) for 3 d in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich). To measure TCR-mediate protein phosphorylation, flow-sorted naïve CD4^{+} T cells were stimulated with plate-coated anti-CD3 and anti-CD28 (2 μg/mL) before being analyzed at indicated time points. In some experiments, cells were treated with either DMSO vehicle or 1 μM GW3965 (Tocris), 1 μM BIO (Sigma-Aldrich), or mevalonic acid (Sigma-Aldrich).

Data, Materials, and Software Availability. The RNAseq data have been deposited to the Gene Expression Omnibus with accession number GSE224303. All study data are included in the article and/or *SI Appendix*.

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