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TGF-beta promotes human T follicular helper cell stemness properties at the expense of effector function

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Introduction: Follicular helper T (T_{FH}) cells are essential for germinal center reactions and the maintenance of long-lived humoral immunity. Transforming growth factor- β (TGF- β) is a multifunctional cytokine implicated in immune regulation, T-cell differentiation, and the maintenance of cellular stemness. Prior studies have shown that TGF- β promotes stemness across a wide range of cell types and facilitates the differentiation of naïve CD4⁺ T cells into various T helper cell subsets. However, its precise effects on T_{FH} effector function and stem-like properties remain poorly understood.

Methods: The dual regulatory roles of TGF- β 1 in modulating T_{FH} effector functions and stem-like properties were investigated using flow cytometry-based phenotyping, co-culture assays with memory B cells, proliferation and apoptosis assays, ELISA for antibody production, and bulk RNA sequencing of naïve-derived and blood-derived T_{FH} cells.

Results: We found that TGF- β 1 treatment in vitro promoted human naïve CD4⁺ T cells differentiation into CXCR3⁺ TFH, but significantly attenuated their effector molecule expression and T_{FH}-mediated memory B-cell differentiation and antibody production, whereas it enhanced the expression of stemness-associated molecules in T_{FH} cells both differentiated in vitro from naïve CD4⁺ T cells and isolated from blood. Notably, TGF- β 1 promoted proliferation and reduced apoptosis of naïve-derived T_{FH} cells in vitro, but suppressed proliferation and increased early apoptosis in blood-derived mature T_{FH} cells.

Discussion: Our findings indicate that TGF- β 1 tunes the balance between T_{FH} effector function and stem-like properties, and show differential regulations of the early phase of T_{FH} differentiation and mature T_{FH} cells, which may have implications for T_{FH}-driven immune pathology and disease.

KEYWORDS

cell differentiation, effector function, stemness properties, Tfh (follicular helper T cells), TGF-beta

Introduction

Follicular helper T (T_{FH}) cells are a distinct subset of $CD4^+$ T cells that play a central role in orchestrating the adaptive immune response. Specifically, they support B cell proliferation, maturation, and antibody production within the germinal centers of secondary lymphoid organs (1, 2). These cells were initially identified by their expression of CXCR5, which allows them to efficiently migrate into B cell follicles and facilitate effective interactions with B cells (3–5). T_{FH} cells perform their functions primarily through the secretion of specific cytokines, such as IL-21, and by engaging with B cell surface molecules, including CD40L and CD40 (6, 7). A comprehensive understanding of the mechanisms that regulate T_{FH} cell differentiation and function is essential for advancing our knowledge of immune responses and for the development of effective therapeutic interventions and vaccines.

Transforming growth factor-beta (TGF- β) is a pleiotropic cytokine that exerts diverse effects on cellular proliferation, differentiation, apoptosis, and immune regulation (8–10). Extensive research has explored its roles in immune cell function, with particular attention to its impact on T_{FH} cells. TGF- β has been shown to modulate T_{FH} cell responses, promote a regulatory phenotype, and influence autoantibody production in autoimmune diseases (11, 12). Moreover, during immune responses to pathogens, TGF- β plays a crucial role in shaping the immune landscape by directing the differentiation of naive $CD4^+$ T cells into T_{FH} cells through the regulation of key transcription factors such as BCL6 and c-MAF (13, 14). Conversely, IL-2-mediated signaling inhibits T_{FH} differentiation (15, 16), and whether TGF- β promotes human T_{FH} differentiation, at least partly, by limiting this pathway also needs to be confirmed.

Numerous studies have demonstrated that TGF- β is instrumental in inducing stemness properties across various cell types. It promotes self-renewal in stem cells by inhibiting differentiation, thereby preserving the stem cell pool (17, 18). Furthermore, TGF- β has been shown to maintain stemness features in multiple biological contexts, including tumor development, hematopoiesis, and immune regulation (19–21). However, under chronic antigen exposure, sustained TGF- β 1 signaling is more closely linked to reinforcing $CD8^+$ T-cell exhaustion than to preserving stem-like potential. Evidence suggests that SMAD2/3-dependent transcription and epigenetic remodeling contribute to stabilizing terminal exhaustion and reducing reinvigoration in response to immune checkpoint blockade (22, 23). Considering the critical roles of T_{FH} cells in immune responses, it is therefore important to investigate whether TGF- β promotes stemness-associated features in T_{FH} cells.

In this study, we investigated the functional characteristics and stemness properties of naive-derived and memory T_{FH} cells in response to TGF- β 1 treatment. Through a combination of cell culture experiments, flow cytometry, and bulk RNA sequencing, we demonstrate that TGF- β 1 enhances stemness-associated transcriptional and phenotypic features in both naive $CD4^+$ T cell-derived T_{FH} cells and circulating memory T_{FH} cells, while simultaneously attenuating their effector functions.

Result

TGF- β 1 promotes human naive $CD4^+$ T cells differentiation into CXCR3 $^+$ T_{FH} cells

To examine the impact of TGF- β on T_{FH} cell differentiation, peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors. To exclude the rare population of stem cell-like T cells displaying a naive phenotype (24, 25), genuine naive $CD4^+$ T cells ($CD3^+ CD4^+ CD45RA^+ CCR7^+ CD95^- CD58^-$) were enriched using magnetic beads and subsequently sorted by flow cytometry. Sorted naive cells were stimulated with anti-human CD3/CD28 beads for 3 days in the presence of various doses of TGF- β 1. T_{FH} cells ($CD4^+ CD45RA^- CXCR5^+$) and their subsets were analyzed by flow cytometry after stimulation (Figures 1A, B). We found that both the proportions and absolute cell numbers of T_{FH} cells derived from naive $CD4^+$ T cells were increased in the presence of TGF- β 1 from 0 to 10 ng/mL in a dose-dependent manner, while declined in the dose of 20 ng/mL (Figure 1C). These data confirm that a low dose of TGF- β 1 efficiently promotes human T_{FH} cell differentiation *in vitro*. However, whether this effect is accompanied by suppression of established negative regulatory pathways, such as the IL-2-STAT5 axis, remains to be determined. Therefore, we first assessed IL-2 secretion levels in the culture supernatant by ELISA to evaluate whether TGF- β 1 affects this negative regulator, and found that IL-2 levels decreased following TGF- β 1 stimulation (Figure 1D). As IL-2 is a key inducer of the STAT5 pathway, the reduction in supernatant IL-2 suggests that TGF- β 1 may further attenuate the IL-2-STAT5 signaling pathway. To directly verify this, T_{FH} cells differentiated from naive $CD4^+$ T cells stimulated with anti-human CD3/CD28 beads, either in the absence or presence of TGF- β 1, were sorted by flow cytometry, and RNA sequencing was performed on these cells. Subsequently, we analyzed pathway activity using GSEA and found that the IL-2-STAT5 pathway was suppressed following TGF- β 1 treatment, with the heatmap showing downregulation of key genes in the pathway, such as IL2RA, STAT5B, PRDM1, and GZMB (Figures 1E, F). In summary, TGF- β 1 treatment not only reduces IL-2 secretion in the culture system but also suppresses IL-2-STAT5 signaling at the transcriptomic level and downregulates its key genes, supporting a model in which TGF- β 1 promotes human T_{FH} differentiation by at least partially antagonizing the IL-2-STAT5 axis.

Human circulating T_{FH} cells can be classified into several subsets based on the expression of CXCR3 and CCR6 (26, 27). Here we found that TGF- β 1 (10 ng/mL) treatment mainly promotes the generation of CXCR3 $^+$ T_{FH} (including T_{FH1} and $T_{FH1/17}$) while significantly inhibits the differentiation of CXCR3 $^-$ T_{FH} cell (including T_{FH2} and T_{FH17}) (Figure 1G). To further evaluate the cytokine profiles associated with subset polarization, IFN- γ levels in the supernatants were measured using ELISA (Figure 1H). The results showed an increasing trend in IFN- γ levels in the supernatants following TGF- β 1 stimulation, further indicating that TGF- β 1 shifts the cell population towards a T_{FH1} -like phenotype. The TGF- β family comprises three isoforms-TGF- β 1, TGF- β 2, and TGF- β 3, which are conserved in mammals (28). Although these isoforms share structural and signaling features,

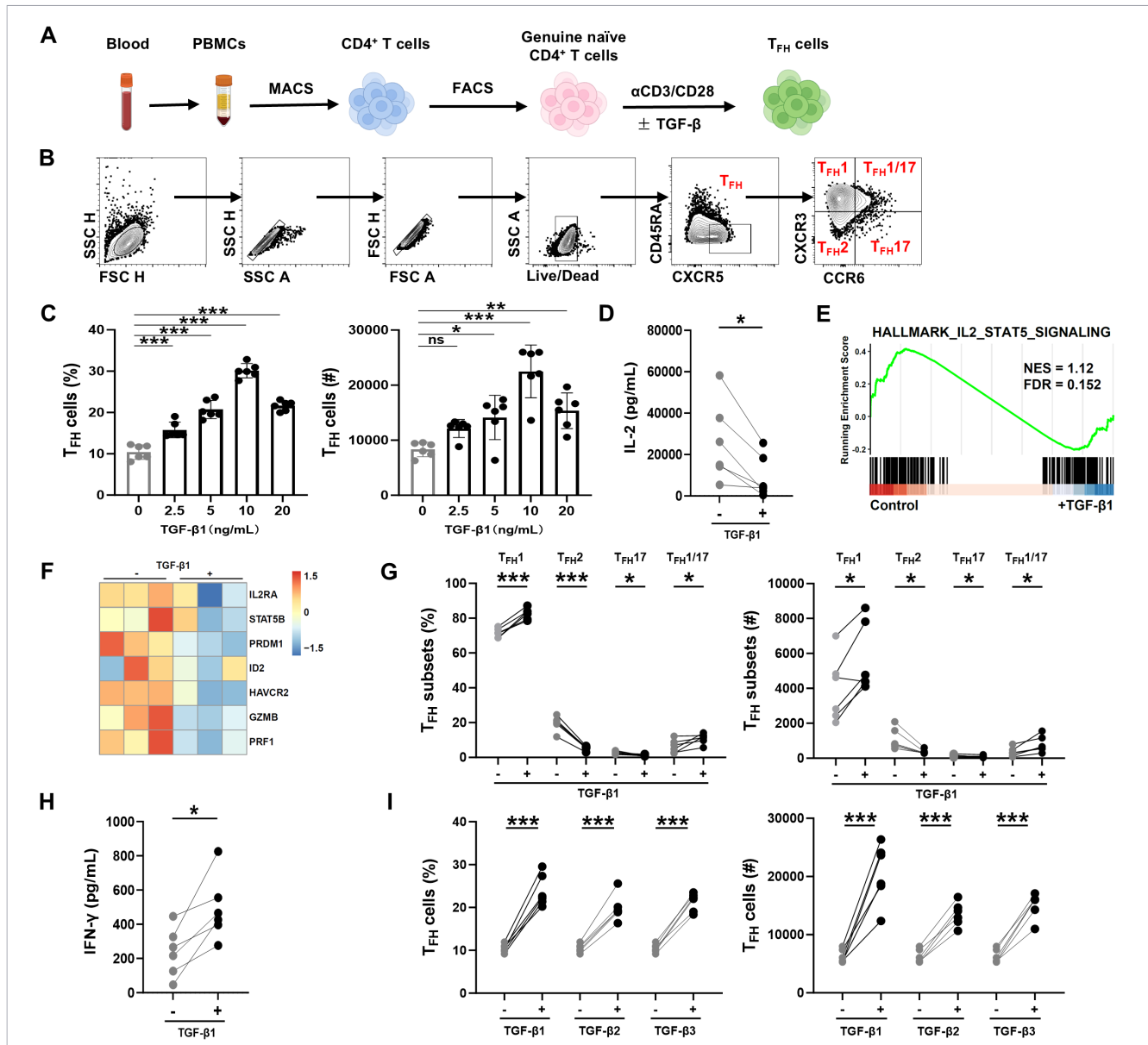


FIGURE 1

TGF- β 1 promotes human naïve $CD4^+$ T cells differentiation into $CXCR3^+$ T_{FH} cells. (A) Schematic of T_{FH} cell differentiation assay. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors, and naïve $CD4^+$ T cells ($CD4^+ CD45RA^+ CCR7^+ CD95^- CD58^-$) were purified and stimulated *in vitro* with anti-CD3/CD28 beads \pm TGF- β 1. (B) Gating strategy for T_{FH} cells ($CD4^+ CD45RA^+ CXCR5^+$) and subsets based on CXCR3/CCR6: T_{FH1} ($CXCR3^+ CCR6^-$), T_{FH2} ($CXCR3^- CCR6^-$), T_{FH17} ($CXCR3^- CCR6^+$), and $T_{FH1/17}$ ($CXCR3^+ CCR6^+$). (C) TGF- β 1 dose-response analysis. Purified naïve $CD4^+$ T cells were stimulated with increasing concentrations of TGF- β 1 (0, 2.5, 5, 10, and 20 ng/mL). The gating was performed according to (B). Bar graphs show T_{FH} cell frequencies and absolute counts (mean \pm SD; $n = 6$ donors). Statistical comparison to 0 ng/mL: one-way ANOVA. (D) IL-2 levels in culture supernatants collected on day 3 from naïve $CD4^+$ T cells stimulated with anti-CD3/CD28 beads in the absence or presence of TGF- β 1 (10 ng/mL), as quantified by ELISA ($n = 6$). (E) Gene Set Enrichment Analysis (GSEA) of RNA-seq profiles from differentiated T_{FH} cells (control vs. TGF- β 1-treated), based on the HALLMARK_IL2_STAT5_SIGNALING gene set (NES = 1.12, FDR = 0.152). (F) Heatmap showing normalized expression of representative genes in the IL-2-STAT5 pathway across individual donors in control and TGF- β 1-treated T_{FH} cells. (G) Subset-specific TGF- β 1 (10 ng/mL). Frequencies and absolute counts of T_{FH} subsets were analyzed. Data represent mean \pm SD ($n = 6$ donors). Statistics: paired t-test per subset vs. untreated. (H) IFN- γ levels in culture supernatants collected on day 3 from the same cultures as in (D), as quantified by ELISA ($n = 6$). (I) Comparative effects of TGF- β isoforms (10 ng/mL). All isoforms increased T_{FH} frequencies/counts similarly (mean \pm SD; $n = 6$ donors). Statistics: paired t-test vs. 0 ng/mL. Each symbol represents one donor with paired samples connected by lines. Significance thresholds: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. $P < 0.05$ was considered to be a two-tailed significant difference, ns, not significant.

they exhibit distinct, context-dependent biological functions (28, 29). To assess the effects of different TGF- β isoforms on T_{FH} differentiation, naïve $CD4^+$ T cells were stimulated with anti-human CD3/CD28 beads in the presence of an equal concentration (10 ng/mL) of TGF- β 1, TGF- β 2, or TGF- β 3. All

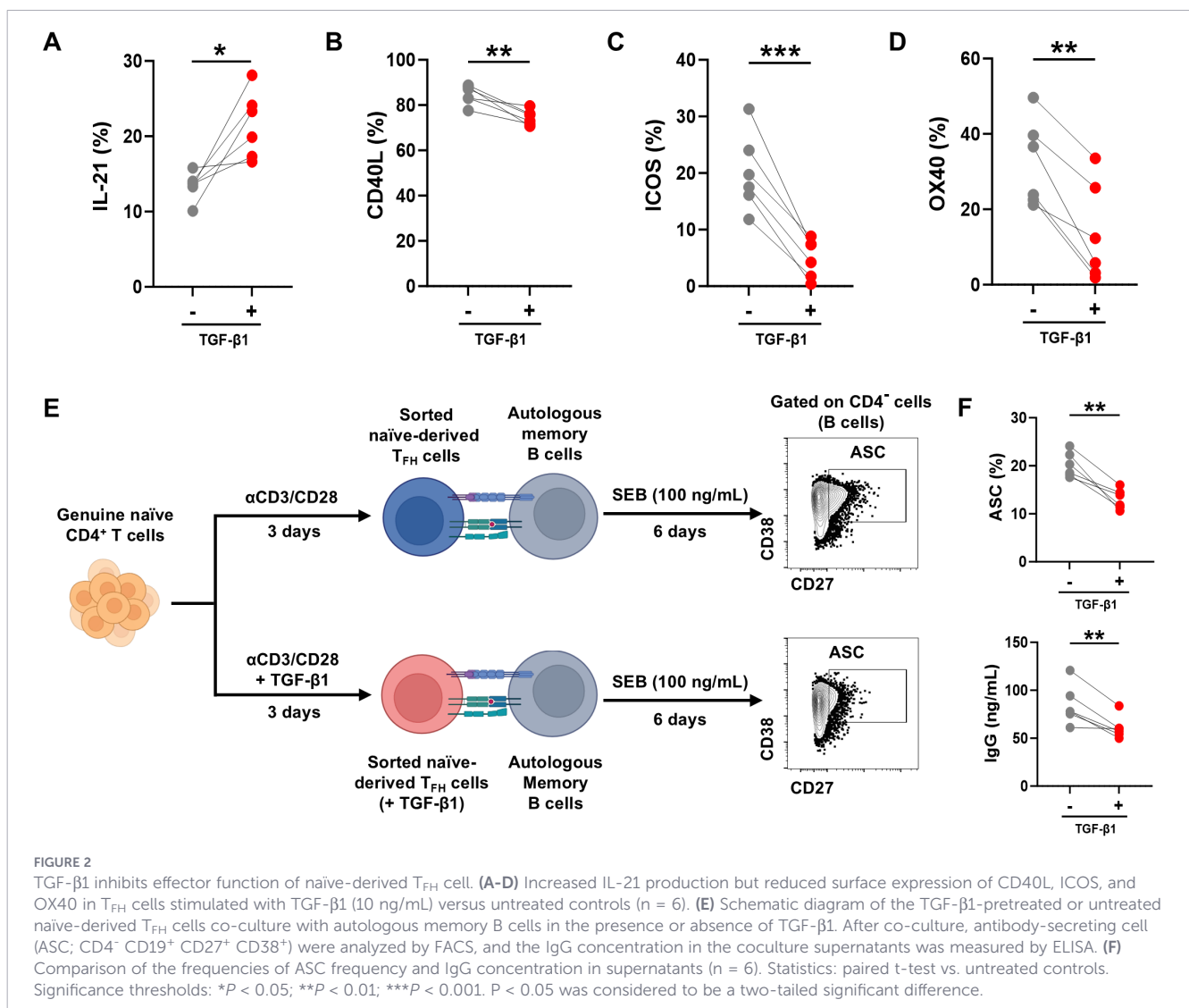
three isoforms promote human T_{FH} cell differentiation in a similar manner (Figure 1I). In summary, TGF- β 1 promotes the differentiation of human naïve $CD4^+$ T cells into T_{FH} cells, at least in part, by suppressing IL-2-STAT5 signaling, and it preferentially drives $CXCR3^+$ T_{FH} differentiation.

TGF-β1 inhibits the effector function of naïve-derived T_{FH} cells

Although TGF-β promotes human naïve CD4⁺ T cell differentiation into T_{FH} cells, whether TGF-β affects the T_{FH} cell function remains to be further explored. T_{FH} cells are characterized by the expression of costimulatory molecules such as ICOS, CD40L, and OX40, and by the secretion of the signature cytokine IL-21, which together are essential for supporting B-cell activation, class switching, and the production of high-affinity antibodies (30, 31). To this end, the expression of CD40L, ICOS, OX40 and IL-21 in naïve-derived T_{FH} cells with or without TGF-β1 (10 ng/mL) was analyzed by flow cytometry. The surface expression of CD40L, ICOS and OX40 was markedly reduced after TGF-β1 treatment, whereas IL-21 production was increased (Figures 2A–D). Given that these T_{FH}-associated molecules are critical mediators of B-cell help, the impact of TGF-β1 on the B-cell helper capacity of T_{FH} cells was further evaluated. First, sorted naïve CD4⁺ T cells were stimulated with anti-

CD3/CD28 for 3 days in the presence or absence of TGF-β1. Subsequently 1×10⁵ naïve-derived T_{FH} cells were sorted by flow cytometry and co-cultured with equal numbers of autologous memory B cells for an additional 6 days in the presence of SEB (100 ng/mL) (Figure 2E). After co-culture, the frequencies of antibody-secreting cells (ASCs; CD4⁺ CD19⁺ CD27⁺ CD38⁺) and the IgG concentrations in culture supernatants were quantified by flow cytometry and ELISA, respectively. Lower proportions of ASCs and reduced IgG levels were observed when memory B cells were co-cultured with TGF-β1-pretreated T_{FH} cells compared with those co-cultured with untreated T_{FH} cells (Figure 2F).

Collectively, these findings indicate that although TGF-β1 promotes the differentiation of naïve CD4⁺ T cells into T_{FH} cells and enhances IL-21 expression, the expression of effector molecules such as CD40L, ICOS, and OX40 in T_{FH} cells was partially impaired by TGF-β1 treatment. Notably, the net impact on helper function of naïve-derived T_{FH} was attenuated by TGF-β1, as evidenced by reduced ASC differentiation and antibody production.



TGF-β1 enhances stemness-associated gene expression, proliferation and survival of naïve-derived T_{FH} cells

It has been reported that TGF-β signaling promotes T-cell longevity and recall capacity by increasing their stemness (21, 32, 33). Here, we confirmed that TGF-β supports human naïve CD4⁺ T cell differentiation into T_{FH} cells *in vitro*, particularly favoring the generation of CXCR3⁺ T_{FH} cells. Concurrently, TGF-β1 treatment impairs their helper function by downregulating effector-associated molecules. However, whether TGF-β modulates the T_{FH} cell stemness-like properties remains undefined. To this end, we analyzed the RNA sequencing data from naïve-derived T_{FH} cells in the presence and absence of TGF-β1. The RNA-seq results

revealed that T_{FH} cells derived from naïve CD4⁺ T cells stimulated with TGF-β1, compared with those without TGF-β1 treatment, exhibited increased transcription of genes associated with T-cell stemness and memory potential, including *IL2RB*, *SELL*, *NOTCH1*, *BCL6*, *FOXO1* and *FOXP1* (Figure 3A). Partial gene expression such as CD122, NOTCH1, BCL6, and FOXO1 was validated by flow cytometry (Figures 3B–E). These data suggest that TGF-β1 enhances T_{FH} cell stemness and memory potential by upregulating stemness or memory associated gene expression.

To determine whether the TGF-β1-imprinted program translates into functional advantages during the maintenance phase, T_{FH} cells were first generated from naïve CD4⁺ T cells by anti-CD3/CD28 stimulation in the absence or presence of TGF-β1. After 3 days, T_{FH} cells were sorted by flow cytometry, and equal

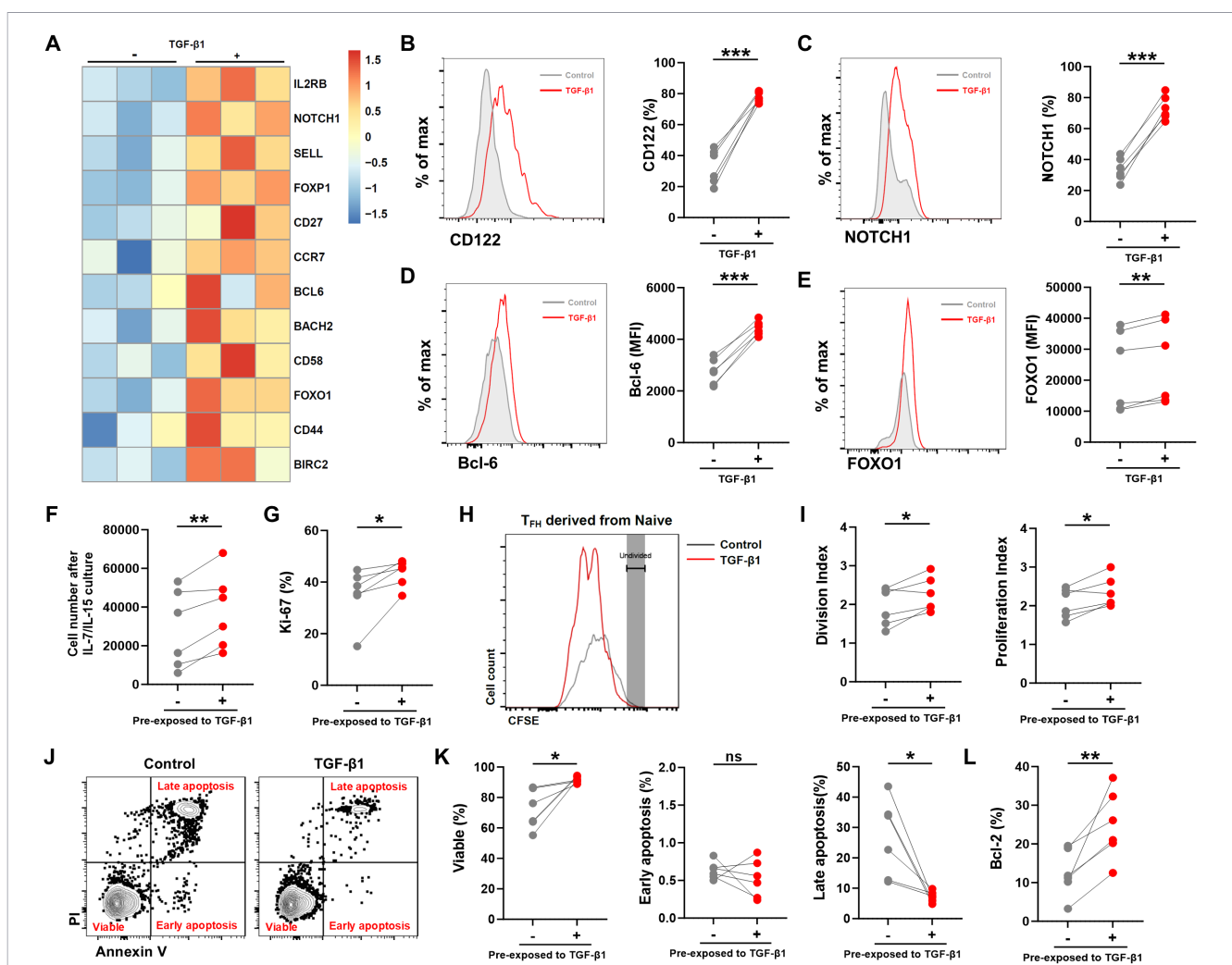


FIGURE 3 TGF-β1 upregulates markers associated with stemness while promoting proliferation and viability in naïve-derived T_{FH} cells. **(A)** Heatmap of stemness-associated genes and their expression in sorted naïve-derived T_{FH} cell populations. **(B–E)** Flow cytometric validation confirming increased protein expression: CD122 (IL2RB), NOTCH1, Bcl-6 (BCL6), and FOXO1 levels (quantified by mean fluorescence intensity, MFI) in naïve-derived T_{FH} cells following TGF-β1 treatment compared to untreated cells; histogram overlays (left) and MFI quantification (right) (n = 6). **(F–I)** Naïve CD4⁺ T cells were stimulated with anti-CD3/CD28 beads in the absence or presence of TGF-β1 (10 ng/mL) for 3 days to generate T_{FH} cells. T_{FH} cells were then sorted by flow cytometry, and equal numbers of sorted T_{FH} cells from each condition were re-plated and cultured with IL-7 and IL-15 for an additional 7 days before analysis. Total cell number after cytokine culture **(F)**, frequency of Ki-67⁺ cells **(G)**, CFSE dilution profile **(H)** and division/proliferation indices **(I)** were assessed at the end of the IL-7/IL-15 culture (n = 6). **(J, K)** Apoptosis was analyzed by Annexin V/PI staining after IL-7/IL-15 culture **(J)** and quantified as indicated **(K)** (n = 6). **(L)** Bcl-2 expression was measured by flow cytometry after IL-7/IL-15 culture (n = 6). Statistics: paired t-test vs. untreated controls. Significance thresholds: *P < 0.05; **P < 0.01; ***P < 0.001. P < 0.05 was considered to be a two-tailed significant difference, ns, not significant.

numbers of cells from each condition were then replated and cultured with the homeostatic cytokines IL-7 and IL-15 for an additional 7 days before analysis. Under these cytokine-driven conditions, T_{FH} cells previously exposed to TGF- β 1 yielded significantly higher cell numbers after culture (Figure 3F), indicating enhanced homeostatic expansion from the same starting input. Consistently, TGF- β 1-conditioned T_{FH} cells showed a higher fraction of Ki-67⁺ cells and increased proliferative expansion (Figures 3G–I).

Consistent with the increased cell recovery, Annexin V/PI staining after IL-7/IL-15 culture showed improved survival in the TGF- β 1 group, reflected by a higher frequency of viable cells (Figures 3J, K). Early apoptosis was not significantly altered, whereas late apoptosis was reduced (Figure 3K). In line with the reduced late apoptosis, TGF- β 1-conditioned T_{FH} cells exhibited an increased frequency of Bcl-2⁺ cells (Figure 3L), supporting a pro-survival effect during cytokine-mediated homeostatic maintenance. Collectively, these data indicate that TGF- β 1 exposure during the initial differentiation phase endows naïve-derived T_{FH} cells with enhanced proliferative capacity and survival during subsequent antigen-independent homeostatic proliferation.

TGF- β 1 also suppresses the B cell helper function of circulating mature memory T_{FH} cells

Memory T_{FH} cells have undergone antigen-driven selection, differentiation, and maturation *in vivo*, and they exhibit robust B cell helper functions (26, 34). To examine the impact of TGF- β 1 on memory T_{FH} cells, circulating memory T_{FH} cells were isolated from PBMCs, stimulated with anti-human CD3/CD28 beads for 3 days in the absence or presence of TGF- β 1. After stimulation, the surface expression of ICOS, CD40L and OX40, as well as intracellular IL-21 production in T_{FH} cells, were analyzed by flow cytometry. The results showed that the surface expression of ICOS, CD40L, and OX40 in T_{FH} cells was markedly reduced, whereas IL-21 production was significantly increased after TGF- β 1 treatment (Figures 4A–D), exhibiting a pattern similar to that observed in naïve-derived T_{FH} cells. To further evaluate the net impact of TGF- β 1 on mature T_{FH} helper function, a co-culture experiment between memory T_{FH} cells and autologous memory B cells was performed. The results showed that TGF- β 1 pretreatment significantly impaired T_{FH} cell helper function, resulting in reduced ASC differentiation and IgG

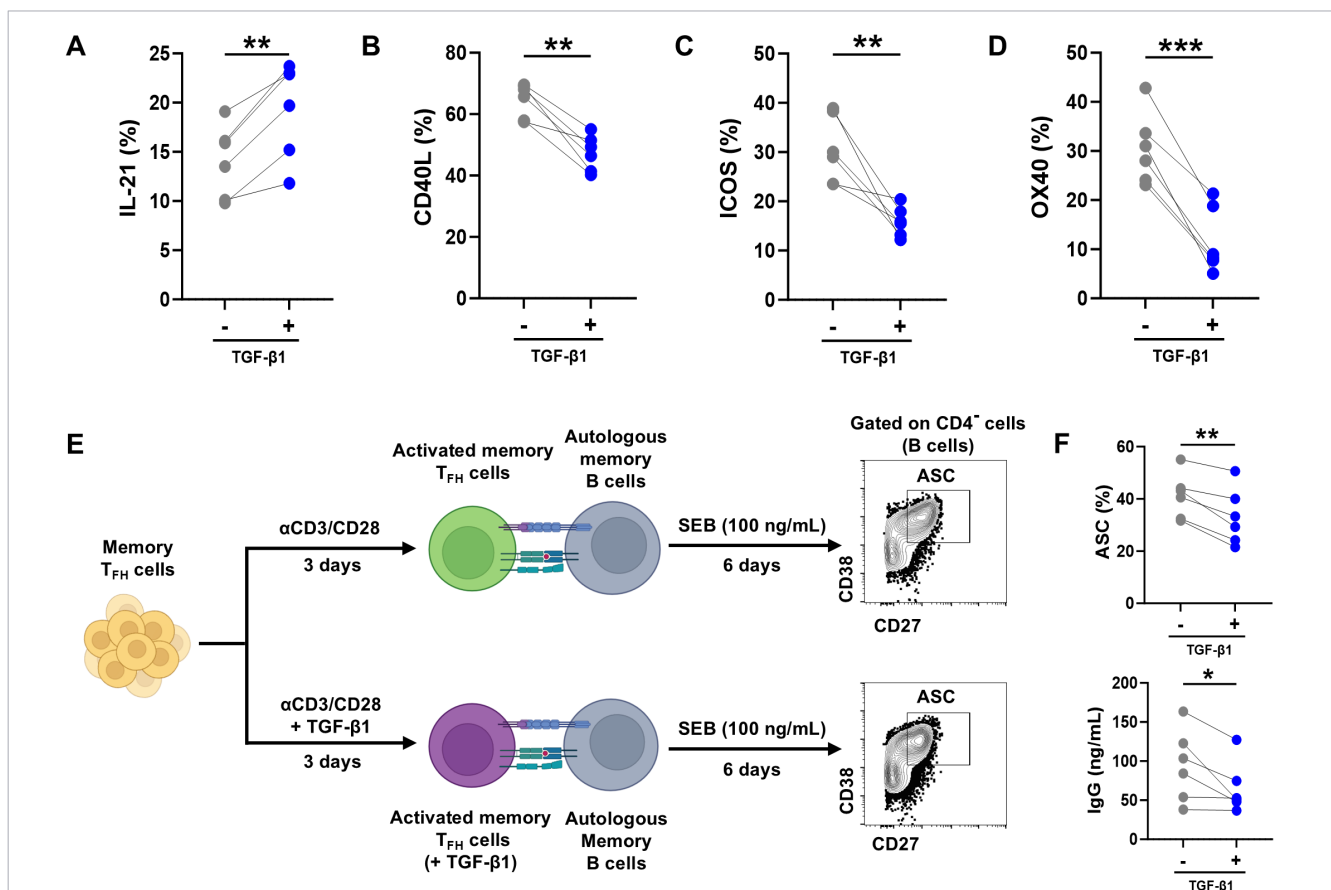


FIGURE 4 TGF- β 1 inhibits effector and helper functions of circulating memory T_{FH} cells. (A–D) Surface expression of ICOS, CD40L, OX40, and intracellular IL-21 production in circulating memory T_{FH} cells isolated from PBMCs and stimulated for 3 days with anti-CD3/CD28 beads \pm TGF- β 1 (10 ng/mL), showing reduced costimulatory molecules but increased IL-21 (n = 6). (E) Schematic diagram: Memory T_{FH} cells pretreated with anti-CD3/CD28 beads \pm TGF- β 1 co-cultured with autologous memory B cells. After co-culture, antibody-secreting cell (ASC; CD4⁺ CD19⁺ CD27⁺ CD38⁺) were analyzed by FACS, and the IgG concentration in the coculture supernatants was measured by ELISA. (F) Comparison of the frequencies of ASC frequency and IgG concentration in supernatants (n = 6). Statistics: paired t-test vs. untreated controls. Significance thresholds: *P < 0.05; **P < 0.01; ***P < 0.001. P < 0.05 was considered to be a two-tailed significant difference.

status of circulating memory T_{FH} cells under the same stimulation conditions. Although the total recovered cell numbers were not significantly altered by TGF- β 1 (Figure 5F), proliferative activity was markedly reduced, as indicated by a decreased frequency of Ki-67⁺ cells and diminished cell division in the presence of TGF- β 1 (Figures 5G–I).

In parallel, Annexin V/PI staining revealed impaired survival upon TGF- β 1 treatment. TGF- β 1 decreased the proportion of viable cells and increased early apoptotic cells, whereas late apoptosis was not significantly changed (Figures 5J, K). Notably, the expression of the anti-apoptotic molecule Bcl-2 remained at comparable levels between groups (Figure 5L), suggesting that TGF- β 1 primarily promotes an early apoptotic phenotype while restraining proliferative expansion in circulating memory T_{FH} cells. Overall, TGF- β 1 induces a stemness- and memory-associated transcriptional and phenotypic program in both naïve-derived and circulating memory T_{FH} cells; however, the functional outcomes are context-dependent. Specifically, TGF- β 1 conditioning enhances the proliferative capacity and survival of naïve-differentiated T_{FH} cells during IL-7/IL-15 maintenance, whereas it limits proliferative expansion and increases early apoptosis in mature memory T_{FH} cells.

Discussion

In this study, we investigated the effects of TGF- β 1 on the differentiation of human naïve CD4⁺ T cells into T_{FH} cells and their subsets, along with its impact on the effector function and stemness properties of both naïve-derived and circulating memory T_{FH} cells. TGF- β 1 was found to promote T_{FH} differentiation, particularly that of the CXCR3⁺ subset, while attenuating T_{FH} effector and B cell helper function. By contrast, TGF- β 1 enhanced stemness-related markers at both the transcriptomic and protein levels. However, its effects on proliferation and survival were cell-phase dependent: it promoted proliferation and survival during the early phase of T_{FH} cell differentiation, but constrained proliferative expansion and increased early apoptosis in mature circulating memory T_{FH} cells. These findings indicate that TGF- β serves as a bidirectional regulator, promoting human T follicular helper cell stemness features at the expense of their effector function, while exerting cell-phase dependent effects on proliferation and survival. This divergence may reflect differences in baseline epigenetic priming, activation history, or the balance of proliferative versus quiescence cues in these two T_{FH} compartments.

TGF- β is a multifaceted cytokine that has been implicated in immunosuppression and the maintenance of cellular stemness within the immune system (37–39). Previous research has shown that TGF- β 1 co-opts with IL-12 or IL-23 promote T_{FH} cell differentiation via signaling STAT3-STAT4 signaling, which are implicated in autoimmune disorders, cancers, and chronic infections (14). Additionally, studies have shown that in T cells, the TGF- β -SMAD2 pathway exerts an antagonistic regulatory effect on the IL-2-STAT5 pathway (40). We confirmed that TGF- β 1 inhibits the activation of this pathway, as reflected by the

downregulation of IL-2 secretion, which may represent one of the mechanisms by which TGF- β promotes T_{FH} differentiation.

Understanding how TGF- β 1 regulates T_{FH} function and stemness properties is crucial for elucidating disease mechanisms and for developing novel therapeutic strategies. In this study, we showed that a series of classical stemness-associated genes, such as SELL, FOXP1, and NOTCH1, were upregulated by TGF- β 1. The upregulation of SELL and FOXP1 may reflect a shift toward a less differentiated, longevity-associated transcriptional state, as SELL has been linked to enhanced persistence and lymphoid-homing capacity of stem-like T cells (41), while FOXP1 functions as a key transcriptional regulator that preserves quiescence and restrains terminal differentiation in multiple T-cell contexts (42). Notably, NOTCH1 was among the most strongly induced genes at both the RNA and protein levels in TGF- β 1-treated T_{FH} populations, suggesting that activation of this pathway may contribute to TGF- β 1-induced plasticity and progenitor-like features of T_{FH} cells. Consistent with this observation, Notch signaling has been reported to be a central regulator of T-cell stemness and memory-like reprogramming (43, 44). In line with a direct role of Notch in T_{FH} biology, T cell-specific deletion of Notch1/2 in murine models has been reported to impair T_{FH} differentiation and germinal center B-cell responses (45). Moreover, cross-talk between the TGF- β and Notch pathways has been shown to contribute to maintaining progenitor-like T-cell states (46, 47). Taken together, these findings support a model in which TGF- β 1 reinforces a stem/progenitor-like transcriptional program and plasticity in both naïve-derived and memory T_{FH} cells, at least in part via induction of Notch signaling, a pathway that is also required for T_{FH} differentiation and productive germinal center B-cell responses. However, the mechanisms by which TGF- β 1 coordinates the regulation of Notch signaling remain to be elucidated.

In addition to direct effects on T cells, upstream myeloid cues may shape a T_{FH1} -like trajectory that could synergize with TGF- β -dependent programming. GM-CSF-activated human CD1c⁺ DCs have been shown to drive naïve CD4⁺ T cells toward T_{FH1} differentiation in a CD40-dependent manner (48). It will be interesting to determine whether TGF- β -rich environments cooperate with such DC-derived polarization to reinforce (or reshape) the CXCR3⁺ T_{FH} state observed in our system.

The preferential induction of CXCR3⁺ (T_{FH1} -like) T_{FH} cells may also have implications beyond humoral immunity, particularly in relation to CD8⁺ T-cell responses. In human breast cancer, functional T_{H1} -oriented T_{FH} cells within TLS are associated with coordinated humoral and cytotoxic immune features, including granzyme-expressing CD8⁺ TILs (49). Consistently, T_{FH} cells can sustain CD8⁺-dependent antitumor immunity via IL-21, and TGF- β has been implicated in shaping a CXCL13 axis that facilitates T_{FH} -CD8⁺ spatial coupling in tumors (50). These studies raise the possibility that a TGF- β -skewed T_{FH1} -like program may coordinate antibody help with CD8⁺ T-cell immunity in specific inflammatory or tumor contexts.

Clinically, targeting TGF- β 1 could help to prevent excessive humoral responses, thereby maintaining immune homeostasis and reducing the risk of autoimmune disease (51). In addition, given its

established role in promoting stem-cell proliferation and differentiation, TGF- β 1 may hold promise for stem-cell therapy and regenerative medicine (52). Moreover, by leveraging TGF- β 1-mediated T_{FH} stemness, vaccines could be designed to maintain T_{FH} cells in a stem-like state, thereby enabling rapid responses upon antigen re-exposure, potentially through optimized antigen delivery and adjuvant selection (53).

TGF- β 1 signaling is commonly divided into canonical SMAD-dependent and non-SMAD pathways. In the canonical pathway, TGF- β 1 activates R-SMADs (e.g., SMAD2 and SMAD3), which form complexes with SMAD4 and translocate to the nucleus, where they regulate genes involved in self-renewal and differentiation, thereby maintaining stemness (54). In contrast, non-SMAD pathways including PI3K-AKT and RAS-MAPK promote stem-cell proliferation and survival, enhance self-renewal, and modulate inflammatory responses and stemness via NF- κ B signaling (55, 56). TGF- β 1 treatment led to marked transcriptional changes related to function and stemness in both naïve-derived and circulating memory T_{FH} cells. Targeting TGF- β 1 signaling may enable therapeutic modulation of T_{FH} function in autoimmune disease and cancer, improve vaccine design for durable responses, and guide regenerative approaches that balance immune activity in autoimmune conditions.

Importantly, not all TGF- β superfamily ligands exert identical effects on T_{FH} function. Activin A has been identified as a potent regulator of human T_{FH}-like differentiation and, in the presence of IL-12, can support a more complete phenotype including robust IL-21 potential (57). This contrasts with the context-dependent effects of TGF- β observed across systems and highlights that shared SMAD2/3 usage does not necessarily translate into shared functional outputs, particularly for key mediators such as IL-21. Dissecting how distinct TGF- β superfamily ligands integrate with the IL-2-STAT5 pathway and inflammatory cues may help explain divergent outcomes in T_{FH} helper function.

However, this study has several limitations. Experiments were primarily conducted *in vitro*, and additional *in vivo* studies are needed to define the effects of TGF- β 1 on T_{FH} cells. Moreover, only transient effects of TGF- β 1 on T_{FH}-subset differentiation were examined. The long-term maintenance of T_{FH} phenotype, function and stem-like properties imprinted by TGF- β 1 requires further investigation.

In conclusion, this study provides evidence that TGF- β 1 promotes human naïve CD4⁺ T cells differentiation into CXCR3⁺ T_{FH} cells and enhances T_{FH} cell stemness-like properties at the expense of T_{FH} cell effector and B-cell helper functions. These findings provide a more comprehensive understanding of the role of TGF- β 1 in regulating T_{FH} cell biological properties, with underlying mechanisms worth further exploration.

Materials and methods

Human cell isolation and sorting

Peripheral blood mononuclear cells were purified from blood samples obtained from volunteers and informed consent was

obtained from all the donors. Cell isolation and sorting were performed by magnetic bead and flow cytometry. Firstly, CD4⁺ T cells and CD19⁺ B cells were isolated from PBMCs by CD4 and CD19 microbeads (Miltenyi Biotec). The purified CD4⁺ T and B cells were further stained with Super Bright™ 600 anti-human CD4 (SK3, eBioscience), PE-eFluor 610 anti-human CXCR5 (MU5UBEE, eBioscience), PerCP/Cyanine5.5 anti-human CD45RA (HI100, BioLegend), FITC anti-human CCR7 (G043H7, BioLegend), PE-Cy7 anti-human CD95 (DX2, BioLegend), PE anti-human CD58 (TS2/9, BioLegend), and PE mouse anti-human CD20 (2H7, eBioscience), PE-Cy7 mouse anti-human CD27 (M-T271, BioLegend), respectively. Stained CD4⁺ T and B cells were sorted by FACS as follows: naïve CD4⁺ T cells (CD95⁻ CD58⁻ CD45RA⁺ CCR7⁺ CD4⁺ T cells), memory T_{FH} cells (CXCR5⁺ CD45RA⁻ CD4⁺ T cells), and memory B cells (CD27⁺ CD20⁺ CD19⁺ B cells).

Stimulation of naïve CD4⁺ T cells and memory T_{FH} cells

Sorted naïve CD4⁺ T cells and memory T_{FH} cells were stimulated with anti-human CD3/CD28 Dynabeads (Invitrogen) in complete RPMI medium (RPMI-1640 medium containing penicillin-streptomycin, 50 μ M 2-mercaptoethanol, 1 mM sodium pyruvate, nonessential amino acids and 25 mM HEPES, pH7.2-7.5) supplemented with 10% fetal bovine serum (FBS) in the absence or presence of the corresponding concentrations of recombinant human TGF- β 1, or TGF- β 2, or TGF- β 3 (PeproTech) for 3 days. Naïve CD4⁺ T cell differentiation/effector function, memory T_{FH} cell effector function was measured by flow cytometry.

Co-culture assay

Naïve CD4⁺ T cells were stimulated with anti-CD3/CD28 Dynabeads in the presence or absence of TGF- β 1 for 3 days. Subsequently, naïve-derived T_{FH} cells were sorted by flow cytometry. A total of 1×10^5 sorted T_{FH} cells were co-cultured with 1×10^5 autologous memory B cells in complete medium for an additional 6 days. Where indicated, staphylococcal enterotoxin B (SEB) was added at 100 ng/mL. At the end of co-culture, antibody-secreting cells (ASCs) were identified by flow cytometry as CD4⁻ CD19⁺ CD27⁺ CD38⁺ cells. IgG concentrations in culture supernatants were measured by ELISA according to the manufacturer's instructions. For experiments using circulating memory T_{FH} cells, purified memory T_{FH} cells were stimulated with anti-CD3/CD28 in the presence or absence of TGF- β 1 for 3 days, after which viable cells were sorted and subjected to downstream analyses as indicated. Cells were isolated from six independent donors. Information regarding age and sex was not available.

Homeostatic maintenance culture and proliferation/survival assays

After 3 days of stimulation, T_{FH} cells were sorted by flow cytometry from the corresponding cultures (for naïve-derived T_{FH}) and from stimulated memory T_{FH} cultures (for memory

T_{FH}), based on the gating strategy described above. Importantly, equal numbers of sorted T_{FH} cells from each condition were replated at the same density and cultured in complete RPMI supplemented with IL-7 (10 ng/mL, KX-PROTEIN) and IL-15 (10 ng/mL, KX-PROTEIN) for an additional 7 days prior to analysis. Total cell numbers after cytokine culture were determined by flow cytometry. For proliferation analysis, sorted T_{FH} cells were labeled with CFSE/CellTrace prior to cytokine culture and CFSE dilution was analyzed after 7 days; division index and proliferation index were calculated in FlowJo (v10.8). For survival/apoptosis analysis, cells were stained with FITC Annexin V and propidium iodide (PI) after IL-7/IL-15 culture and the frequencies of viable, early apoptotic, and late apoptotic cells were quantified.

Antibody staining and flow cytometry

Surface staining was performed for 30 minutes at 4 °C in phosphate-buffered saline containing 2% FBS. The fluorescently labeled antibodies used were as follows: Super Bright™ 600 anti-human CD4 (SK3, eBioscience), PerCP/Cyanine5.5 anti-human CD45RA (HI100, BioLegend), PE-eFluor 610 anti-human CXCR5 (MU5UBEE, eBioscience), PE anti-human CD40L (24-31, BioLegend), PE/Cy7 anti-human ICOS (C398.4A, BioLegend), APC/Cy7 anti-human OX40 (Ber-ACT35, BioLegend), PE anti-human CD38 (HB-7, BioLegend), PE/Cy7 anti-human CD27 (M-T271, BioLegend), Brilliant Violet 421™ anti-human CD122 (TU27, BioLegend), PE-Cy7 anti-human CD95 (DX2, BioLegend), FITC anti-human CCR7 (G043H7, BioLegend). To measure intracellular IL-21 secretion, cultured and purified T_{FH} cells were stimulated with phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (1 µg/mL) for 6 h, brefeldin A (BioLegend) added during the final 4 h. For intracellular staining, cells were fixed and permeabilized using a fixation/permeabilization kit (BD Biosciences), followed by staining with PE anti-human IL-21 (3A3-N2, BioLegend), BV650 anti-human Notch1 (MHN1-519, BD Biosciences), PE-Cy7 anti-human Ki-67 (Ki-67, BioLegend), Alexa Fluor® 488 anti-human Bcl-2 (100, BioLegend). For nuclear staining, cells were processed with the fixation/permeabilization concentrate (eBioscience), and subsequently stained with PE anti-human FOXO1 (W20064D, BioLegend), APC anti-human Bcl-6 (BCL-UP, eBioscience), Alexa Fluor® 647 anti-human TCF-1 (7F11A10, BioLegend). The samples were acquired on a CytoFLEX SRT Flow Cytometer (Beckman Coulter) immediately after antibody staining. Gating strategies were established using fluorescence minus one (FMO) controls and unstained controls. Data were analyzed with FlowJo v10.8 software (TreeStar).

Enzyme-linked immunosorbent assay

For IL-2 and IFN- γ quantification, culture supernatants were clarified by centrifugation when necessary and stored at ≤ -20 °C (or -80 °C) until analysis. IL-2 and IFN- γ levels were measured using the Human IL-2 and IFN- γ ELISA MAX™ Deluxe Set (BioLegend) following the manufacturer's protocol. Briefly, 96-well plates were coated with diluted capture antibody overnight at 2-8 °C, washed,

and blocked with Assay Diluent A for 1 h at room temperature (with shaking). Standards and appropriately diluted samples were then added for 2 h, followed by incubation with biotinylated detection antibody for 1 h and Avidin-HRP for 30 min. After washing, TMB substrate was added for color development, and the reaction was stopped with stop solution; absorbance was read at 450 nm within 15 min, and concentrations were calculated from the standard curve.

The concentration of IgG in the co-culture supernatants was determined using a human IgG ELISA kit (Mabtech) according to the manufacturer's instructions. Briefly, 96-well plates pre-coated with anti-human IgG capture antibody were incubated with serially diluted IgG standards and appropriately diluted culture supernatant samples for 2 hours at room temperature. After washing, plates were incubated with HRP-conjugated anti-human IgG detection antibody, followed by the addition of TMB substrate solution. The colorimetric reaction was stopped with 2 N H₂SO₄, and absorbance was measured at 450 nm using a microplate reader. IgG concentrations were calculated based on the standard curve generated from known IgG concentrations.

Bulk RNA-sequencing

Bulk RNAseq was performed on 1×10^5 purified naïve-derived T_{FH} cells and memory T_{FH} cells after cultured with anti-human CD3/CD28 beads in the presence or absence of 10 ng/mL TGF- β 1 for 3 days. Purified T_{FH} cells were fully dissolved with 1 mL of Trizol (Takara) and sent to GENEWIZ (Suzhou, China) for library construction and sequencing analysis. Libraries were prepared using standard Illumina protocols and sequenced on a NovaSeq 6000 platform (Illumina) to generate 150 bp paired-end reads. Raw reads were processed using Cutadapt (v1.9.1) to remove adapter sequences and low-quality bases. Clean reads were aligned to the human reference genome (GRCh38) using HISAT2 (v2.2.1), and gene-level counts were quantified with HTSeq (v0.6.1). Further analyses were performed with R software (v4.4.1). Raw read counts were normalized, and differential expression was analyzed using the Bioconductor package DESeq2 (v1.6.3). Heatmaps were generated with the package pheatmap (v1.0.12). GSEA was calculated with the package ClusterProfiler (v4.12.6).

Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 10.1; GraphPad Software). Data are shown as mean \pm SEM unless otherwise indicated. Comparisons between two groups were made using the paired two-tailed t-test, while multiple group comparisons were performed using one-way ANOVA followed by Tukey's *post hoc* test. Statistical significance was defined as $p < 0.05$.

Data availability statement

RNA-seq data presented in the study are deposited in the GEO repository (<https://www.ncbi.nlm.nih.gov/geo/>), accession number GSE320556.

Ethics statement

The studies involving humans were approved by Medical Ethics Committee of University of South China. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

YY: Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. CW: Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing. WY: Visualization, Writing – review & editing. LP: Validation, Writing – review & editing. YZ: Visualization, Writing – review & editing. XZ: Data curation, Methodology, Writing – review & editing. YW: Resources, Writing – review & editing. KJ: Supervision, Writing – review & editing. WL: Conceptualization, Funding acquisition, Resources, Writing – original draft, Writing – review & editing.

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Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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