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TGF-β co-opts STAT3-STAT4 signaling to promote human T follicular helper cell differentiation

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Abstract

Understanding the developmental mechanisms of T follicular helper (T_{FH}) cells in humans is a highly relevant topic to clinic. However, factors that drive human $CD4^+$ helper T (T_H) cell differentiation program towards T_{FH} cells remain largely undefined. Here we show that TGF-β provides critical additional signals for the transcription factors STAT3 and STAT4 to promote the initial T_{FH} differentiation programs in humans. This mechanism does not appear to be shared with mouse T_H cells. The developing human Bcl-6^+ T_{FH} cells also expressed RORγt, a transcription factor typically expressed by T_H17 cells. Our study documents a mechanism by which T_{FH} and T_H17 cells co-emerge in inflammatory environments in humans, as often observed in many human autoimmune diseases.

T follicular helper (T_{FH}) cells play a major role in the generation of antigen-specific antibody responses by providing help to B cells[1]. T_{FH} cells are essential for the formation of germinal centers (GCs), where high-affinity B cells are selected and differentiate into long-lived memory B cells and plasma cells[2]. The chemokine receptor CXCR5 is expressed by T_{FH} cells and guides their migration towards B cell follicles[1]. T_{FH} cells highly express the inducible co-stimulatory molecule ICOS, which is critical for their development[3, 4], migration into follicles[5] and function[6]. T_{FH} cells support the survival of GC B cells and their differentiation into memory cells and plasma cells through secretion of interleukin 21 (IL-21)[7] and by providing signals through the TNF family receptor superfamily molecule CD40[1]. While T_{FH} cells are important for antibody responses against infectious agents, exaggerated T_{FH} responses cause autoimmunity[8]. Therefore, defining the developmental mechanism of T_{FH} cells in humans is a highly relevant topic to human pathophysiology, and would provide direct insights into designing novel vaccines for infectious diseases and developing novel therapeutic approaches for autoimmune diseases.

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Author contribution:

N.S. conceived and executed the experiments, and analyzed the data. Y.L and L.B. performed the tonsillar tissue staining. S.E.B. contributed to the preparation of tonsillar Th cells. I.M. performed NanoString assay. K.V. contributed to the experiments with mouse cells. H.U. oversaw and conceived the entire project, and analyzed the data. N.S., J.B., and H.U. wrote the manuscript.

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T_{FH} precursors interact with B cells at the border of T cell zone and follicles. Prolonged and stable interactions with B cells are essential for their maturation into GC T_{FH} cells[1, 9]. Nonetheless, dendritic cells (DCs) are important in the early stage of T_{FH} cell generation. The programing of $CD4^+$ helper T (T_H) cell towards T_{FH} cell differentiation occurs as early as the first few divisions following interaction with DCs[4, 9, 10]. DC-derived cytokines activating the transcription factors STAT3[11] and STAT4[12] induce the interacting Th cells to express Bcl-6, a transcriptional repressor essential for T_{FH} maturation[13, 14, 15]. The function of Bcl-6 is inhibited by the transcriptional repressor Blimp-1, and accordingly Blimp-1 inhibits the generation of T_{FH} cells[13]. ICOS ligand expressed by DCs also contributes to the expression of Bcl-6 in T_H cells[4]. Therefore, encounter with DCs largely pre-determines whether T_H cells differentiate into the T_{FH} lineage[9].

Similar to other T_H subsets, cytokine signals are important for the early development of T_{FH} cells. Previous studies suggest differences between humans and mice regarding the dominant cytokines involved in T_{FH} cell development. In mice, IL-6, IL-21 and IL-27 (that activate STAT3) play dominant roles[1, 16], while IL-12 (that mainly activates STAT4) can also participate in the early phase[12]. In contrast, IL-12 appears to be more important than IL-6, IL-21, and IL-27 for T_{FH} cell generation in humans[17, 18]. IL-12 induces higher expression of IL-21, ICOS, CXCR5 and Bcl-6 on activated human naïve T_H cells compared with the other cytokines[18, 19]. However, IL-12 is also implicated in the generation of T_H1 cells, suggesting that additional factors may also contribute to the generation of human T_{FH} cells. How STAT4 and STAT3 signaling contributes to the generation of human T_{FH} cells also remains to be established.

Here we show that TGF-β is an important co-factor for the early differentiation of human T_{FH} cells. TGF-β co-operated with IL-12 and IL-23 for the expression of multiple T_{FH} molecules by human naïve T_H cells including CXCR5, ICOS, IL-21, Bcl-6, BATF and c-Maf, and the downregulation of Blimp-1. This stimulatory effect of TGF-β for T_{FH} development was not found in mice. In the presence of TGF-β, STAT4 and STAT3 shaped the human T_H differentiation gene programs towards the T_{FH} lineage in a largely redundant manner, and cooperated to induce the expression of T_{FH} molecules. Furthermore, we found that human T_H17 cells generated in vitro with the cytokine combination of IL-23+IL-6+IL-1β+TGF–β largely shared properties with T_{FH} cells, suggesting that the early developmental path of T_{FH} and T_H17 cells is shared in humans. We also found T_{FH} cells co-expressing Bcl-6 and RORγt in human tonsils, providing supportive evidence for co-development of T_{FH} and T_H17 cells in inflammatory environment in humans.

Results

TGF–β cooperates with IL-12 and IL-23 for T_{FH} molecule expression

We took a systematic approach to determine the cytokine signals promoting the initial T_{FH} differentiation programs in humans. We cultured adult blood naïve T_H cells from 13 different donors with CD3-CD28 mAbs in the presence of different cytokine combinations for 2–4 days, and analyzed the expression of multiple molecules expressed by T_{FH} cells, including CXCR5, Bcl-6, ICOS and IL-21. For this analysis we selected cytokines known to regulate T cell differentiation, many of which are secreted by DCs: IL-1β IL-6, IL-10, IL-12,

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IL-21, IL-23, type I interferons (IFN-α, β and ω), type III IFNs (IFN-λ1, λ2) and TGF-β To circumvent the direct inhibitory effect of TGF-β and IFNs on cell cycle, naïve T_H cells were primed overnight with CD3-CD28 mAbs before addition of cytokines to the cultures. This procedure yielded comparable cell recovery and viability across different cytokine conditions (Supplementary Fig. 1a). The data obtained from naïve T_H cells cultured with various cytokines were ranked and the cytokine conditions that increased the expression of each T_{FH} molecule were determined. Of note, we excluded IL-4, a major driver for T_H2 differentiation, from the analysis, because previous studies suggested that IL-4 by itself does not induce human naïve Th cells to express T_{FH} molecules[18, 19]. We further found that IL-4 strongly inhibited the expression of ICOS and IL-21 in IL-12-stimulated naïve Th cells (Supplementary Fig. 1b,c) and as such IL-4 might negatively regulate the generation of human T_{FH} cells.

Analysis of the expression of CXCR5 by activated ($FSC^{hi}SSC^{hi}$) human naïve T_H cells cultured with various cytokines for 3 days showed that the highest expression of CXCR5 was induced by the cytokine combination of TGF-β+IL-23 (Fig. 1a,b**, and** Supplementary Fig. 1d). The stimulation with TGF-β+IL-23 and TGF-β+IL-12 (also together with IL-1β, IL-6, IL-10, and IL-21) induced higher expression of CXCR5 than the stimulation with IL-23 or IL-12 alone. The cytokine conditions containing TGF-β+IL-23 and TGF-β+IL-12 also promoted the expression of ICOS and IL-21 (Fig. 1a–c). In contrast, type I and III IFNs yielded low expression of CXCR5 and ICOS, and few IL-21^+ T cells (Fig. 1a–c). T_H cells cultured with TGF–β+IL-12 and TGF–β+IL-23 efficiently induced B cells to produce IgG *in vitro*, while T_H cells cultured with TGF–β or TGF–β+IL-6 did not (Fig. 1d). Thus, the combination of TGF-β+IL-12 and TGF-β+IL-23 promoted the expression of T_{FH} markers and induced efficient B cell help.

TGF–β+ IL-12 and TGF–β+ IL-23 induce Bcl-6 in human naïve T_H

To assess the early T_H differentiation programs at a transcriptional level, we analyzed the expression of transcription factor transcripts by NanoString nCounter® Analysis System, which allows direct measurement of mRNA transcript abundance. Similar to mouse T_{FH} cells[1, 16], we confirmed that human ex vivo tonsillar GC T_{FH} cells and their precursors[7] have high expression of *BCL6*, *BATF* and *MAF*, but low expression of *PRDM1* (encoding Blimp-1; Supplementary Fig. 2a,b). We assessed the expression of these transcription factors in human adult blood naïve T_H cells cultured for 3 days with different cytokines as described above. The data obtained were normalized to the values in the control culture (no cytokines) in each experiment, and the ranking of the cytokine conditions that determined. TGF-β+IL-12 and TGF-β+IL-23 induced higher *BCL6* expression than IL-12 or IL-23 alone (Fig. 2a). Furthermore, addition of IL-1β and IL-6 to TGF-β+IL-12 and TGF-β+IL-23 further increased *BCL6* expression. TGF-β+IL-12 and TGF-β+IL-23 also suppressed the expression of *PRDM1*. IL-1β and IL-6 further decreased *PRDM1* expression when added to TGF-β+IL-12. Immunoblotting showed that protein expression correlate with the transcript data (Fig. 2b). Furthermore, overall *PRDM1* abundance negatively correlated with *BCL6* abundance (Fig. 2c), and accordingly, the combinations of TGF-β+IL-12+IL-1β+IL-6 yielded the highest ratio of *BCL6* to *PRDM1* (Supplementary Fig. 2c). Largely similar

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results were obtained with human cord blood-derived naïve T_H cells (Supplementary Fig. 2d).

The stimulation of human naïve T_H cells with the combinations of TGF-β+IL-12 and TGF-β +IL-23 together with IL-1β and IL-6 also resulted in high expression of *BATF* and *MAF* (Supplementary Fig. 3a). In particular, the abundance of *BCL6* showed a strong positive correlation with the abundance of *BATF* (R=0.90 in adult blood naïve T_H and R=0.84 in cord blood naïve T_H; Fig. 2c **and** Supplementary Fig. 3b). Kinetic analysis showed that the cytokine combinations of TGF-β+IL-12+IL-1β+IL-6 and TGF-β+IL-23+IL-1β+IL-6 as well as TGF-β+IL-12+IL-23 increased the expression of *BCL6* and *BATF* within 24 h (Fig. 2d). While *BATF* expression peaked at 24 h after cytokine stimulation, *BCL6* expression peaked at 96–120 h, suggesting that BATF directly induces Bcl-6 expression in human T_H cells, as shown in mice[20, 21]. Chromatin immunoprecipitation experiments indicated that BATF binds to promoter regions of the human *BCL6* gene (Fig. 2e, Supplementary Fig. 3c). Furthermore, the combinations of TGF-β+IL-12 and TGF-β+IL-23 together with IL-1β and IL-6 enhanced the expression of *JUN* and *JUNB*, two transcription factors which form functional heterodimers with BATF[22] (Supplementary Fig. 2d). The abundance of *JUN* and *JUNB* also showed a strong positive correlation with the abundance of *BCL6* (R=0.91 and 0.88, respectively; Fig. 3a). These results suggest that TGF-β+IL-12 and TGF-β+IL-23 together with IL-1β and IL-6 promote Bcl-6 expression at least in part by upregulating both components of BATF-Jun heterodimers.

To address whether TGF-β also promotes the expression of T_{FH} markers in naïve T_H cells primed under physiological conditions, human adult blood naïve T_H cells were stimulated with allogeneic monocyte-derived DCs (activated either by heat-killed *E. coli* or CD40L stimulation) in the presence of titrated amounts of TGF-β. The expression of CXCR5, IL-21 and Bcl-6 was enhanced by a supplementation of 0.2–1 ng/ml of TGF-β (Supplementary Fig. 3d,e), consistent with the results obtained with anti-CD3-CD28-stimulated T_H cells.

Collectively, TGF-β+IL-12 and TGF-β+IL-23 together with IL-1β and IL-6 promoted human naïve T_H cells to express multiple T_{FH} molecules including CXCR5, ICOS, IL-21, Bcl-6, BATF-Jun, and c-Maf, while downregulating Blimp-1.

T_H cells at proximity of GCs receive TGF–β signaling

These results suggest that when naïve T_H cells interact at T cell zone with activated DCs that produce inflammatory cytokines including IL-12 and IL-23[7], TGF-β promotes T_H differentiation towards the T_{FH} lineage. T_H cells in the proximity of tonsillar GCs are known to express phosphorylated STAT4 (p-STAT4), suggesting that T_H cells receive IL-12 signals at these sites[17]. We examined whether TGF–β is also expressed in the T cell zone of inflammatory pediatric tonsils. We observed abundant expression of TGF–β in the T cell zone near GCs, in particular in the proximity of $CD31^+$ lymphatic and vascular endothelial cells (Fig. 3a). More than 50% of the $CD11c^+$ DCs localized in the T cell zone were positive for TGF–β staining, indicating that T_H cells have access to environmental TGF–β and/or TGF–β expressed by DCs.

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TGF–β mediates its biological functions by binding to TGF–β type I and type II receptors, which phosphorylate the transcription factors Smad2 and Smad3. Activated Smad molecules translocate into the nucleus[23]. To determine whether T_H cells receive TGF–β signals in the T cell zone, we analyzed the expression of phosphorylated Smad2 (p-Smad2) in human pediatric tonsils. p-Smad2 was expressed in many cells in the inflamed tonsils, including endothelial cells (Fig 3b), consistent with abundant expression of TGF–β. T_H cells in the proximity of GCs (determined by DAPI staining pattern. not shown) showed p-Smad2 nuclear expression (Fig. 3b) and p-STAT4 expression (Fig. 3c) These observations indicate that the T cell zone in inflamed tonsils is enriched in TGF–β, and that T_H cells in the proximity of GCs receive TGF–β and IL-12 signals.

T_{FH}-promoting conditions induce T_H17 transcriptional signatures

The observations that the IL-23+IL-1β+IL-6+TGF-β cytokine combination, which is commonly used for the generation of human Th17 cells i*n vitro*[24], was one of the most efficient combinations tested at inducing *BCL6* expression in naïve T_H cells (derived from both adult peripheral blood and cord blood samples) suggested that the initial differentiation process of human T_H17 cells might be shared with T_{FH} cells. In this context, the global abundance of *BCL6* in the cultured adult blood naïve T_H cells was found to positively correlate with the abundance of the T_H17-associated transcription factors *AHR*, *RORA* and *RORC* (encoding RORγt) (Fig. 4A). In contrast, *BCL6* abundance negatively correlated with the abundance of *GATA3, FOXP3* and *IKZF4* (encoding Eos). Largely similar results were obtained in experiments with cord-blood naïve T_H cells, except *TBX21* (encoding T-bet) which did not show any correlation with *BCL6* (Supplementary Fig. 4). Thus, the conditions promoting the T_{FH} differentiation also increased the expression of T_H17-associated transcription factors, but decreased the expression of T_H2- or T_{reg} cell-associated transcription factors.

An unsupervised clustering of the culture conditions according to the transcription factors expressed by adult naïve T_H cells cultured for 3 days with various cytokines (indicated in Fig. 2a) revealed 4 clusters (hereafter called cluster I, II, III and IV; Fig. 4b). The pattern of the transcription factor expression in Clusters III and IV was largely similar, and represented a T_{FH} transcriptional signature (such as upregulation of *BCL6*, *BATF* and *MAF*, and downregulation of *PRDM1*). Clusters III and IV also represented a T_H17 transcriptional signature (characterized by the upregulation of *AHR*, *RORA* and *RORC*, while *GATA3* expression was substantially diminished). The culture conditions that induced the transcriptional signatures characteristic to Clusters III and IV included TGF-β+IL-12 and TGF-β+IL-23 with or without IL-1β and/or IL-6. A T_{reg} transcriptional signature (upregulation of *FOXP3* and *IKZF4*) was dominant in Cluster I and found in conditions including TGF–β alone and combinations of TGF–β with each of the following: IFNs, IL-1β, IL-6, IL-10 and IL-21. This signature was absent in Clusters III and IV. Cluster II did not show strong transcriptional signatures of either T_{FH}, T_H17 or Tregs, and was characteristic of stimulatory conditions that lacked TGF–β. These results suggest that the culture conditions that promoted T_{FH} transcriptional signatures also promoted T_H17 transcriptional signatures, but not T_H2 or Treg transcriptional signatures.

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Bcl-6$^+$ developing T_{FH} cells co-express RORγt

We next determined whether the developing T_{FH} cells share properties with T_H17 cells, or these two subsets emerge separately. Adult blood naïve T_H cells stimulated with CD3-CD28 mAbs were cultured under no cytokines, IL-12, IL-23, TGF-β+IL-12 (with or without IL-1β and/or IL-6), and TGF-β+IL-23 (with or without IL-1β and/or IL-6, and thus including the T_H17 condition IL-23+TGF–β+IL-6+IL-1β[24]), and analyzed for single cell expression of Bcl-6, T-bet, and RORγt by flow cytometry. Kinetics analysis showed that TGF–β+IL-12 and TGF–β+IL-23 increased the expression of Bcl-6 and RORγt, which peaked 2–3 days after the addition of cytokines (meaning at day 3–4 of culture; Fig. 5a,b). T-bet expression was decreased by TGF–β+IL-12 and TGF–β+IL-23 compared to IL-12 and IL-23 alone, respectively (Fig. 5b, Supplementary Fig. 5a).

We next determined the expression of Bcl-6, T-bet, and RORγt of CXCR5$^+$ TH cells differentiated in various cytokine conditions. Whereas CXCR5$^+$ T_H cells differentiated with IL-12 or IL-23 alone expressed little Bcl-6, more than 60% of CXCR5$^+$ T_H cells differentiated with TGF–β+IL-12 and TGF–β+IL-23 expressed Bcl-6 (Fig. 5c, Supplementary Fig. 5b). Within the CXCR5$^+$ T_H cells differentiated with TGF–β+IL-12 and TGF–β+IL-23, more than 70% of RORγt$^+$ cells co-expressed Bcl-6. The differentiation of CXCR5$^+$Bcl-6$^+$RORγt$^+$ T_H cells required IL-12 and IL-23, because TGF–β+IL-6 induced CXCR5$^+$ T_H cells expressing RORγt, but not Bcl-6 (Supplementary Fig. 5c). siRNA-mediated knock down of RORγt in T_H cells cultured in the T_H17 condition IL-23+TGF–β +IL-6+IL-1β increased the development of RORγt$^-$Bcl-6$^+$ cells, confirming that RORγt was not essential for expression of Bcl-6 (Supplementary Fig. 5d). Furthermore, IL-17A$^+$ cells differentiated by culturing cord-blood naïve Th cells in the T_H17 condition IL-23+TGF–β +IL-6+IL-1β expressed both RORγt and Bcl-6 (Fig. 5d), and approximately 40% of IL-17A$^+$ T_H cells differentiated in this condition co-expressed IL-21 (Supplementary Fig. 5e).

Collectively, these results show that TGF–β+IL-12 and TGF–β+IL-23 induced CXCR5$^+$ T_H cells co-expressing Bcl-6 and RORγt.

Both STAT3 and STAT4 cooperate with TGF–β in T_{FH} differentiation

Whereas IL-12 and IL-23 activate both STAT4 and STAT3 in human T_H cells, STAT4 in IL-12 signaling and STAT3 in IL-23 signaling deliver the major activation signals[25]. We next determined whether STAT4 and STAT3 play distinct or redundant roles in expression of T_{FH} and T_H17 transcriptional signatures in human adult blood naïve T_H cells cultured with TGF–β+IL-12 and TGF–β+IL-23 (as shown in Cluster III and IV in Fig. 2b). Human adult blood naïve T_H cells were first transfected with specific siRNA to downregulate the expression of STAT4 and STAT3[18], and cultured with TGF–β+IL-12 and TGF–β+IL-23. siRNA-transfected naïve T_H cells were also cultured with combinations of TGF–β with each of STAT3-activating cytokines IL-6, IL-10 and IL-21. For the analysis, the abundance of each transcript analyzed in STAT3 and STAT4-siRNA transfected T_H cells was normalized separately against data obtained from scrambled siRNA-transfected cells cultured in the same cytokine conditions. Knock down of STAT3 in T_H cells cultured with combinations of TGF–β with each of IL-23, IL-21 and IL-6, and knock down of STAT4 in T_H cells cultured with TGF–β+IL-12 resulted in a largely similar TF modulation pattern, in which the T_{FH}

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transcriptional signature (characterized by high *BCL6*, *BATF* and *MAF*, and low *PRDM1*) as well as the T_H17 transcriptional signature (high *AHR*, *RORA*, and *RORC*) were inhibited, while T_H2 (*GATA3*) and Treg (*FOXP3* and *IKZF4*) transcriptional signatures were enhanced. Of note, STAT3 knock down in T_H cells cultured with IL-12+TGF-β also resulted in downregulation of T_{FH} and T_H17 signatures, and upregulation of T_H2 and Treg signatures, supporting the importance of STAT3 in the T_{FH} programing of IL-12-stimulated human T_H cells[26]. Thus, in the presence of TGF–β signaling, STAT3 and STAT4 play redundant roles to drive human T_H differentiation gene programs towards T_{FH} and T_H17 cells, and away from Treg and T_H2 cells.

A fraction of tonsillar T_{FH} cells express RORγt and T-bet

Our observations suggest the existence of human T_{FH} cells that express RORγt. However, previous studies concluded that human tonsillar $CXCR5^{hi}ICOS^{hi}$ GC T_{FH} cells do not express RORγt, because *RORC* mRNA expression in GC T_{FH} cells was substantially lower than in T_H17 cells[27]. Analysis of mRNA transcripts showed that human ex vivo tonsillar $CXCR5^{hi}ICOS^{hi}$ GC T_{FH} cells expressed modest, yet more abundant *RORC* mRNA than $CXCR5^{neg}ICOS^{neg}$ naïve T_H cells (Supplementary Fig. 6a), suggesting that a subset of $CXCR5^{hi}ICOS^{hi}$ GC T_{FH} cells might express RORγt. To address this, we analyzed the expression of Bcl-6 and RORγt in single human tonsillar T_{FH} cells by flow cytometry and confocal microscopy. Flow cytometry analysis showed that approximately 25% of $CXCR5^{hi}ICOS^{hi}$ Bcl-6^+ GC T_{FH} cells expressed RORγt (Fig. 7a). As expected, backgating of Bcl-6^+ cells showed that they were largely confined to the $CXCR5^{hi}ICOS^{hi}$ GC T_{FH} subset among tonsillar T_H cells (Fig. 7b). Analysis by confocal microscopy confirmed that Bcl-6^+ T_H cells were largely limited within GC (Supplementary Fig. 6b,c). Importantly, backgating of Bcl-6^+RORγt^+ T_H cells revealed that these cells were also confined to $CXCR5^{hi}ICOS^{hi}$ GC T_{FH} cells (Fig. 7b). In contrast, RORγt^+ cells lacking the expression of Bcl-6 were largely confined to $CXCR5^{lo}ICOS^{hi}$ T_H subset (Fig. 7b), which are inefficient B cell helpers[7]. Furthermore, by confocal microscopy, T_H cells expressing RORγt were found both outside and within the GCs (Fig. 7c,d).

We also analyzed whether human tonsillar GC T_{FH} cells contained subsets of cells expressing T-bet or GATA-3. GC T_{FH} cells expressed more abundant *TBX21* mRNA than naïve T_H cells (Supplementary Fig. 6a). In contrast, *GATA3* mRNA expression was similar between GC T_{FH} cells and naïve T_H cells. Flow cytometry analysis revealed that a fraction of Bcl-6^+ GC T_{FH} cells co-expressed T-bet, but not GATA3 (Supplementary Fig. 6d). Thus, human tonsillar Bcl-6^+ GC T_{FH} cells contain cells co-expressing RORγt and T-bet, but not GATA3. Given that the microenvironment of inflamed tonsils is enriched in TGF-β and inflammatory cytokines, including IL-12 (Fig. 3), these observations supports the observation that the combination of TGF–β and STAT3- and STAT4-activating cytokines induces naïve T_H cell differentiation programs towards T_{FH} and T_H17 lineage, but away from T_H2 cells.

TGF-β suppresses ICOS and IL-21 expression by mouse T_H cells

The significance of TGF-β for the generation of T_{FH} cells *in vivo* can be potentially tested in mouse models. However, a previous study in mouse models showed that blocking TGF-β

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did not decrease the expression of CXCR5 on activated T_H cells or the generation of GC B cells[28]. Furthermore, multiple studies with mouse T_H cells show that TGF–β inhibits the expression of Bcl-6[14, 28] (partly via promoting the expression of miR-10a that suppresses Bcl-6 expression[29]) and IL-21[30]. These observations suggest that TGF-β might inhibit the generation and/or the function of T_{FH} cells in mice. To directly address whether the effect of TGF-β on T_{FH} development is different between humans and mice, mouse naïve T_H cells were first primed overnight with CD3-CD28 mAbs (as with human naïve T_H cells), and then cultured with combinations of titrated amounts of TGF-β with IL-6+IL-21 (commonly used to generate mouse T_{FH}-like cells *in vitro*[31]), IL-12+ IL-6+IL-21, and IL-23+ IL-6+IL-21. Consistent with previous studies[30], while promoting IL-17A expression, TGF-β dose-dependently inhibited IL-21 expression by mouse T_H cells cultured under any tested cytokine combinations (Fig. 8a,b; Supplementary Fig. 7). Furthermore, TGF-β also dose-dependently suppressed the expression of ICOS. CXCR5 expression by T_H cells was minimal under any culture conditions (consistent with previous studies[32]), and TGF-β did not substantially affect its expression. These results show that the effect of TGF-β on the expression of T_{FH} molecules differs between mice and humans, and that the positive regulation by TGF-β for the generation of T_{FH} cells appears to be limited to humans.

Discussion

Previous studies suggested that IL-12 is important for T_{FH} cell generation in humans[17, 18]. Individuals (particularly children) deficient for the IL-12 receptor β1 (IL-12Rβ1) chain have reduced T_{FH} and GC responses, providing *in vivo* evidence that signals via IL-12 receptor are essential for the generation of T_{FH} cells in humans[17]. However, IL-12 stimulation by itself seems insufficient, because Bcl-6 expression in IL-12-stimulated human naïve T_H cells is much lower than in tonsillar T_{FH} cells[17]. Furthermore, whereas IL-12Rβ1 is shared by receptors for IL-12 and IL-23, whether IL-23 also contributes to the generation of human T_{FH} cells was unknown. Our present study demonstrates that both IL-12 and IL-23 contribute to human T_{FH} generation, and TGF–β acts as a critical co-factor of IL-12 and IL-23 for the T_{FH} cell differentiation in humans. The T_{FH} transcriptional signature (that is upregulation of Bcl-6, c-Maf and BATF; downregulation of Blimp-1) was strongly induced when TGF–β was combined with IL-12 and IL-23. IL-23, but not by IL-12, promoted the expression of Blimp-1. However, TGF–β strongly diminished the capacity of IL-23 to induce Blimp-1 expression. In inflammatory lymphoid organs, TGF–β was abundantly expressed in the T cell zone, where naïve T_H cells interact with activated DCs. The presence of T_H cells co-expressing p-Smad2 and p-STAT4 adjacent to GCs provided evidence that developing T_{FH} cells receive signals that activate both pathways. We propose that an integration of these signals promote human naïve T_H cells to undergo T_{FH} differentiation gene programs along with the upregulation of multiple T_{FH} molecules. Such initial differentiation process likely promotes their migration towards follicles and the interactions with B cells to differentiate into mature T_{FH} cells[9].

Our study shows that the cooperation of TGF–β and STAT3-STAT4 cytokines occurs in at least two aspects of human T_{FH} differentiation. First, while lacking this capacity by itself, TGF–β enhances the function of STAT3-STAT4 to induce human naïve T_H cells to express T_{FH} molecules, including CXCR5, ICOS, IL-21, Bcl-6, BATF-Jun and c-Maf. Importantly,

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this stimulatory effect of TGF-β for T_{FH} development seems limited to human T_H cells, and not shared with mouse T_H cells. Enhanced Bcl-6 expression in human naïve T_H cells cultured with TGF–β+IL-12 and TGF–β+IL-23 together with IL-1β and IL-6 was at least partly mediated by increased BATF-Jun expression. Second, TGF–β suppresses, and STAT3-STAT4 further downregulates (in the presence of TGF–β), Blimp-1 expression in human naive T_H cells. Because TCR stimulation is sufficient to induce human T_H cells to express Blimp-1, but not Bcl-6[33], inhibition of Blimp-1 expression likely represents an important mechanism to shift the Bcl-6 versus Blimp-1 balance towards Bcl-6 dominance, and thus to promote the T_{FH} differentiation.

In contrast to TGF-β, type I and III IFNs were found to inhibit T_{FH} cell differentiation by inhibiting the establishment of a T_{FH} transcriptional signature, as well as the expression of T_{FH} molecules. Type I IFN signals were recently shown to inhibit the development of T_{FH} cells in mice *in vivo*[34], suggesting that this mechanism is shared between mice and humans. These observations would suggest that exaggerated T_{FH} response in type I IFN-mediated human autoimmune diseases, such as systematic lupus erythematosus (SLE)[35], is not caused by the direct effect of type I IFNs on T_H cells, but by an indirect effect on other immune cells, such as DCs. This hypothesis is further supported by mouse studies demonstrating that the adjuvant effect of type I IFNs for antibody response is solely mediated by IFN-responsive DCs[36, 37].

The combination of TGF–β and STAT3-STAT4 induced in human naïve T_H cells a differentiation program directed towards the T_{FH} and T_H17 lineages, and away from the T_H2 and Treg lineages. In this context, STAT3 and STAT4 contributed in a largely similar fashion, and complemented each other. IL-23+TGF–β+IL-6+IL-1β, the most common cytokine cocktail for the generation of human T_H17 cells *in vitro,* was one of the most efficient cytokine combinations to induce Bcl-6 expression. T_H17 cells expressing RORγt and IL-17A generated with this cytokine cocktail co-expressed T_{FH} molecules such as CXCR5, ICOS, IL-21, and Bcl-6, indicating that developing T_H17 cells can share properties with T_{FH} cells. This does not appear to be limited to developing cells, as human tonsillar Bcl-6^+ GC T_{FH} cells contained a subset that co-expressed RORγt. In addition, blood memory T_{FH} cells also contain a subset sharing properties with T_H17 cells[38]. This blood memory T_{FH} subset can be defined by the co-expression of CXCR5 and CCR6, and is more efficient at providing help to B cells than $CCR6^-$ blood T_{FH} subset[38, 39]. Importantly, the frequency of $CCR6^+$ blood T_{FH} subset was found to be increased in patients with various autoimmune diseases including juvenile dermatomyositis[38], Sjogren's syndrome[40] and multiple sclerosis[41]. These observations suggest that T_{FH} subset sharing properties with T_H17 cells represents more efficient B cell helpers than other subsets, and an increase of these cells is associated with the development of human autoimmunity. Furthermore, both T_{FH} and T_H17 cells co-emerge in many human autoimmune diseases, including SLE[42], rheumatoid arthritis[8], Sjogren's syndrome[43], multiple sclerosis[44] and juvenile dermatomyositis [38]. The shared developmental mechanism for T_{FH} and Th17 cells provides a strong mechanistic insight for their co-emergence in these diseases. Given that TGF–β is also abundantly expressed in inflammatory sites in human autoimmune diseases[23, 45] where

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tertiary lymphoid organs are often formed, it is presumable that TGF–β-rich inflammatory sites also contribute to the generation of T_{FH} and T_H17 cells.

In conclusion, our study demonstrates that TGF–β promotes T_{FH} response in inflammatory environment by collaborating with STAT3 and STAT4-activating cytokines. Our conclusion will provide insights in the pathogenesis of human autoimmune diseases. Establishing the global transcriptional network promoting the human T_{FH} cell differentiation program and the expression of multiple T_{FH} molecules, in a comprehensive approach as shown in mouse T_H17 cells[46], might identify novel therapeutic target molecules for their treatment.

Online Methods

Isolation of naïve T_H cells

For human cells, the study was approved by the Institutional Review Board of Baylor Research Institute. PBMCs were purified from apheresis blood samples obtained from adult volunteers and cord blood samples obtained at Baylor Health Care System. Informed consent was obtained from all the donors. Naïve T_H cells were first enriched by negative selection with purified CD8 (HIT8a), CD11b (LM1/2), CD11c (B-ly6), CD14 (M5E2), CD15 (W6D3), CD16 (3G8), CD19 (J4.119), CD45RO (UCHL1), CD56 (C218) and HLA-DR (B8.12.2) mAbs, and Dynabeads Pan Mouse IgG (Dynal). The mouse study was approved by Institutional Animal Care and Usage Committee of Baylor Research Institute. For isolation of mouse naïve T_H cells, splenocytes were isolated by mechanical disruption of spleen harvested from C57BL/6 female mice (6 to 10 weeks old). Mouse T_H cells were then enriched by negative selection with the T_H cell isolation kit II (Miltenyi). Naive T_H cells were further purified by sorting with FACSAria (BD Biosciences) as $CD4^+$ $CCR7^+$ $CD45RA^+$ $CD8^-$ $CD56^-$ HLA-DR^- for human and as $CD4^+$ $CD62L^{high}$ $CD44^{low}$ $B220^-$ $CD8^-$ $CD11b^-$ $CD11c^-$ $CD25^-$ for mouse. Cell purity was >99%.

Tonsillar cells

Tonsil samples were obtained from healthy subjects undergoing tonsillectomies, and single cells were collected by mechanical disruption. B cells were removed with CD19 MACS Microbeads (Miltenyi Biotech). The tonsillar T_H subsets were sorted from $CD8^-CD19^-CD56^-CD4^+$ cells according to their different level of CXCR5 and ICOS expression[7]. Tonsillar GC T_{FH} cells express CXCR5 and ICOS at high levels ($CXCR5^{hi}ICOS^{hi}$). T_H cells expressing CXCR5 and ICOS at low levels ($CXCR5^{lo}ICOS^{lo}$) show phenotypic and functional similarities with GC T_{FH} cells, yet localize outside GCs (Pre-T_{FH} cells)[7]. Tonsillar $CXCR5^{neg}ICOS^{neg}$ Th cells are largely constituted by naïve T_H cells, while $CXCR5^{lo}ICOS^{hi}$ T_H cells are enriched with IL-17A-secreting cells [7]. Cell purity was >98%.

Stimulation of naïve T_H cells with CD3-CD28 mAbs

After overnight stimulation of naïve T_H cells with anti-human or mouse CD3-CD28 Dynabeads (Invitrogen) in RPMI complete medium supplemented with 10% FCS, cells were transferred to flat-bottomed 96 well plates coated with CD3 mAb (5 μg/ml, clone OKT3 for human and 145-2C11 for mouse) and supplemented with soluble CD28 mAb (1 μg/ml, clone

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CD28.2 for human and 37.51 for mouse) and the following human (h) or mouse (m) recombinant cytokines: hIL-1β (10 ng/ml), hIL-4 (10 ng/ml), hIL-6 (25 ng/ml), hIL-10 (10 ng/ml), hIL-12 (1 ng/ml), hIL-21 (25 ng/ml), hIL-23 (25 ng/ml), hTGF-β (5 ng/ml unless indicated), hIFN-α (50 IU/ml), hIFN-β(10 ng/ml), hIFN-λ1 (10 ng/ml), hIFN-λ2 (10 ng/ml), hIFN-ω (10 ng/ml), mIL-12p70 (10ng/ml), mIL-23 (20ng/ml), mIL-6 (20ng/ml) and mIL-21 (50ng/ml). Cell viability was determined by trypan blue staining (Vi-Cell Cell Viability Analyzer. Beckman Counter).

Stimulation of naïve T_H cells with DCs

Monocyte-derived DCs were generated as described previously[18]. Briefly, Monocytes were isolated from adult PBMCs by negative selection using Monocyte Isolation Kit II (Miltenyi Biotec). DCs were generated by culturing monocytes with 50 ng/ml IL-4 (R&D) and 100 ng/ml GM-CSF (Leukine) in RPMI complete medium supplemented with 10% FCS in 6 well plates (2×10^6 cells/3 ml/well). Cytokines were added every 2 days. At day 6, DCs were stimulated with irradiated CD40L-transfected L-cells or heat killed *Escherichia coli*. After 5 hours stimulation, DCs were harvested and carefully washed. Allogeneic naïve T_H cells (4×10^4 cells/well) were cultured for 4 d with activated DCs (8×10^3 cells/well) loaded with SEB (0.2 μg/ml) in the presence of titrated amounts of TGF-β in 96 well round bottom plates. The phenotype and the cytokine expression profiles were analyzed by flow cytometry.

Flow cytometry

Cultured human T_H cells were stained with CXCR5 AF647 (RF8B2) and ICOS biotin (ISA-3) mAbs + Streptavidin PerCP. For intranuclear staining, cells were fixed with Cytofix buffer (BD) for 10 min at 37 °C and incubated with Bcl6 (K112-91), RORγt (AFKJS-9) and T-bet (O4-46) mAbs or isotype control in Perm/wash buffer I (BD) for 30 min at room temperature. For the analysis of intracytoplasmic IL-21, cultured T_H cells or tonsillar T_{FH} cells were stimulated with PMA (25 ng/ml) and ionomycin (1 μg/ml) for 6 h in the presence of GolgiStop (BD Biosciences) and Brefeldin (eBioscience) for the last 4 h. Human T_H cells were stained with CXCR5 mAb and then for intracytoplasmic cytokines with IL-21 (3A3-N2) and IL-17A (BL168). Cultured mouse naïve T_H cells were stained with CXCR5 (L138D7) and ICOS (C398.4A). IL-21 expression in mouse T_H cells was detected as previously reported[30]. Briefly, permeabilized T cells were incubated sequentially with IL-21R/Fc, PE conjugated F(ab′)2 goat anti-human IgG antibody and then with IL-17A (TC11-18H10.1) antibodies. All the samples were incubated with LIVE/DEAD fixable Aqua to exclude dead cells from the analysis. Cells were acquired on a BD FACS Canto II or a BD LSRII. Expression of each molecule was assessed with FlowJo software (TreeStar) in each tonsillar T_{FH} subsets or for cultured cells in activated ($FSC^{high}SSC^{high}$) T_H cells.

Nanostring

Freshly isolated or cultured T_H cells were lysed in RLT buffer. Total RNA was purified using RNeasy Micro Kit (Qiagen). The NanoString reactions were done according to manufacturer's instructions, and the data were normalized to multiple housekeeping genes included in the codeset. The analysis of ex vivo tonsillar T_H subsets included probes for

Bcl-6 cofactors *CTBP1* (encoding CtBP), *BCL6B* (encoding BAZF), *MTA3*, *NCOR2* (encoding SMRT), *BCOR*, *ZBTB17* (encoding MIZ1), *RUNX1T1* (encoding ETO), and *ZBTB16* (encoding PLZF).

siRNA transfection

After overnight stimulation of naïve T_H cells with CD3-CD28 Dynabeads, cells were transfected with siRNA using the Human T cell Nucleofector Kit and Nucleofector II device (Amaxa) according to manufacturer's instructions. siRNA to target STAT3 (s743), STAT4 (s13531) and silencer select negative control #1 siRNA (Ambion) were used at 5 μM (0.5 nmol/5 × 10^6 cells/transfection)[18]. Six hours post-transfection, the cells were transferred to flat-bottomed 96 well plates coated with CD3 mAb in presence of soluble CD28 mAb. The indicated cytokines were added to the culture at 24 h post-transfection.

Immunoblotting

Whole cell extract were prepared using RIPA buffer. Equal amounts of total protein were electrophoresed on 4%–12% Bis-Tris gels (Invitrogen), transferred to PVDF membrane, and blotted with the following antibodies: anti-Bcl6 (clone D-8, Santa-Cruz), anti-Blimp1 (clone 6D3, Santa-Cruz) or Peroxidase-conjugated anti-GAPDH (clone GAPDH-71.1, Sigma). Peroxidase-conjugated Goat anti-mouse or Goat anti-rat antibodies (Jackson ImmunoResearch) were used for detection and specific bands were visualized with ChemiDoc™ MP System. Band intensity was quantified and normalized to the GAPDH loading controls.

Chromatin Immunoprecipitation

After 3 days culture of naïve T_H cells with IL-23+IL-1β+IL-6+TGF-β, the cells were crosslinked with formaldehyde treatment and the chromatin was fragmented to 200 to 500 bp by sonication. Each ChIP experiment was performed on chromatin from 2 × 10^7 cells. The chromatin fraction was incubated overnight with 5 μg of anti-BATF (Brookwood Biomedical) or Rabbit IgG (Invitrogen) linked to protein A Dynabeads (Invitrogen). Immune complexes were washed and protein-DNA cross-links were reversed for 4 hours at 65°C. The samples were purified using ChIP DNA purification kit (Active Motif), resuspended in 50μl of Tris-EDTA and subsequently used for SYBR Green real-time PCR amplification. The primers were designed to cover two BATF binding sites predicted with MatInspector Professional (Genomatix) within the promoter region of *BCL6* gene. ChIP-qPCR Human IGX1A Negative Control (Qiagen) was used for a negative control. The percentage of total genomic input was determined.

T-B coculture assay

B cells were enriched from apheresis PBMCs by positive selection using CD19 MicroBeads and LS column (Miltenyi Biotec). Memory B cells were sorted with FACSAria as $CD27^+$ $CD3^-$ $CD11c^-$ $CD14^-$ cells, after staining with CD27 PE (L128), CD3 APC (SK7), CD11c APC (S-HCL-3), and CD14 APC (TüK4) mAbs. Cell purity was >98%.

Activated FSC^{high} T_H cells after 3 d culture with the indicated cytokines were sorted and co-cultured with autologous memory B cells (2.5 ×10^3 T cells for 40 × 10^3 memory B cells per

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well) in 96-well U-bottom plates in Yssel medium/10% FBS in the presence of endotoxin-reduced SEB (0.25 ng/ml; Toxin technology, Inc.). The amounts of IgG produced in the cultures were quantified by ELISA at day 14.

Immunofluorescence

6 μm-frozen sections from tonsils fixed with cold acetone were stained with TGF-β (TB21, AbD Serotec), CD31 (Rabbit polyclonal, Abcam) and CD11c (S-HLC-3, BD) antibodies followed by anti–mouse IgG1 conjugated to A568, anti-Rabbit A488 and anti-mouse IgG2b A647, respectively. Finally, sections were counterstained for 2 min with 3 μM of the nuclear stain DAPI. Slides were mounted with Fluoromount G (Southern Biotec) and observed under Nikon Ti-E Inverted microscope with NIS Elements software and Coolsnap HQ2 camera using Planapo 4x/0.2 and Planapo 20/0.75 objectives.

Confocal microscopy

6 μm cryostat sections were fixed for 10 min in cold acetone and permeabilized with 0.1 % Triton X100 for 10 min. After treatment with Fc Receptor Blocker and Background Buster (Innovex Biosciences) for 30 min each, slides were incubated overnight with primary antibodies at 4°C. For the pSmad2, CD3, CD11c staining, fixed sections were stained with Rabbit polyclonal anti-pSmad2 (Cell Signaling), mouse IgG2a anti-CD3 (HIT3a, BD Bioscience) and mouse IgG2b anti-CD11c (S-HLC-3, BD) antibodies followed by anti-Rabbit A488, anti–mouse IgG2a A568, and anti–mouse IgG2b A647, respectively. For the pSmad2, pSTAT4, CD3 staining, the primary staining was done with rabbit polyclonal anti-pSmad2, mouse IgG1 anti-pSTAT4 (E-2, Santa Cruz) and mouse IgG2a anti-CD3 (HIT3a, BD) followed by a secondary staining with goat anti-rabbit A488, anti-mouse IgG1 A568 and anti-mouse IgG2a A647. For the RORγt, IgD, CD4 staining, the tissue section was first incubated with polyclonal rabbit anti RORγt (Abcam), mouse IgG2a anti-IgD (IA6-2, BD) and mouse IgG1 anti-CD4 (RPA-T4, BD) followed by incubation with anti-rabbit A568, anti-mouse IgG2a A488 and anti-mouse IgG1 A647. For the Bcl-6, IgD, CD4 staining, the tissue section was first incubated with mouse IgG1 anti-Bcl-6 (PG-B6P, Abcam), rabbit anti-IgD (Dako) and mouse IgG2b anti-CD4 (OKT4, Biolegend) then secondarily stained with anti-mouse IgG1 A568, anti-Rabbit A488 and anti-mouse IgG2b A647. Finally, sections were counterstained for 2 min with 3 μM of the nuclear stain DAPI, and mounted with prolong gold antifade reagent (Invitrogen). The slide images were observed under a Leica SP5 confocal microscopy with 203/0.7.403/1.25, and 633/1.4 Planapo Objectives.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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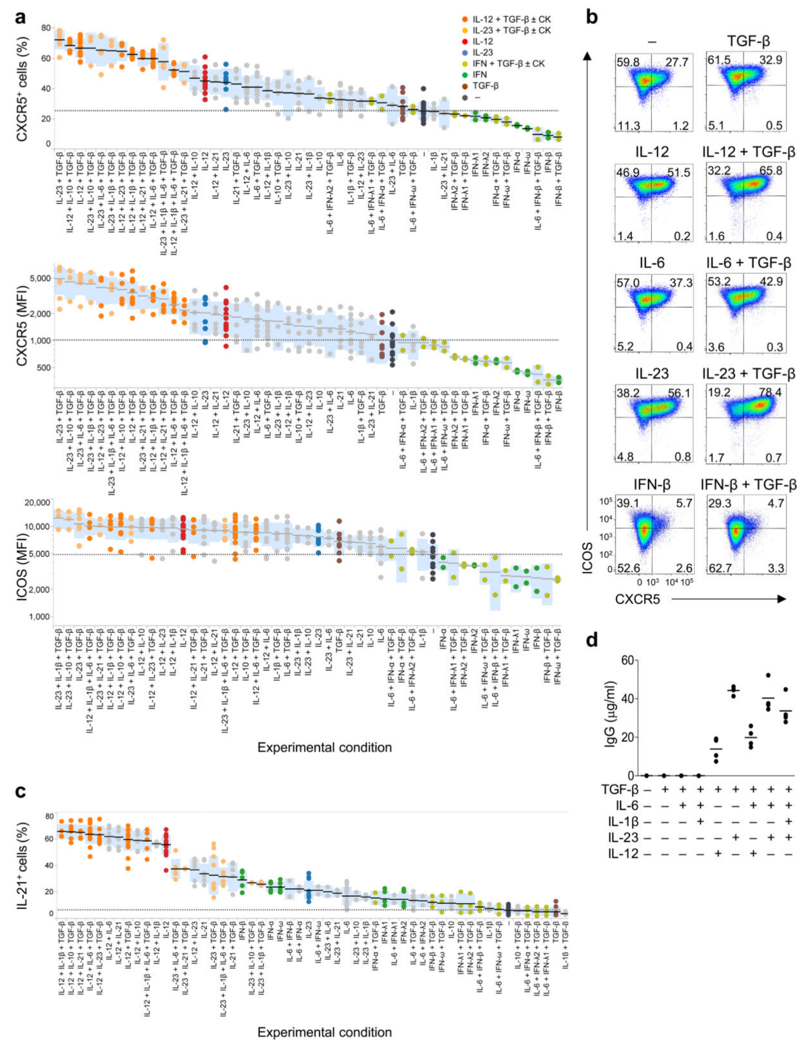

Fig. 1. TGF–β+IL-12 and TGF–β+IL-23 induce T_{FH} molecule expression by human naïve T_H cells

(a) Ranking of the differentiation conditions inducing the expression of CXCR5 and ICOS on activated ($FSC^{hi}SSC^{hi}$) human adult blood naïve T_H cells following 4 d stimulation with CD3-CD28 mAbs in the presence of the indicated cytokines (CK) during the last 3 days. Each dot represents a result from 13 sets of 4 d-culture experiments. Black bars show the mean value and the blue boxes show the range of ± 1 s.d. **(b)** Expression of CXCR5 and ICOS on activated T_H cells stimulated with the indicated cytokines as in **(a)**. Results are from one representative of 13 sets of 4 d-culture experiments. **(c)** Ranking of the differentiation conditions inducing IL-21 expression by activated human naïve T_H cells cultured as in **(a). (d)** IgG ELISA at day 14 in the supernatants of co-cultures of activated naïve T_H cells (after 3 d culture with the indicated cytokines) and autologous memory B cells in the presence of the superantigen Staphylococcal enterotoxin B. Mean, n=4. A representative experiments out of 3 experiments.

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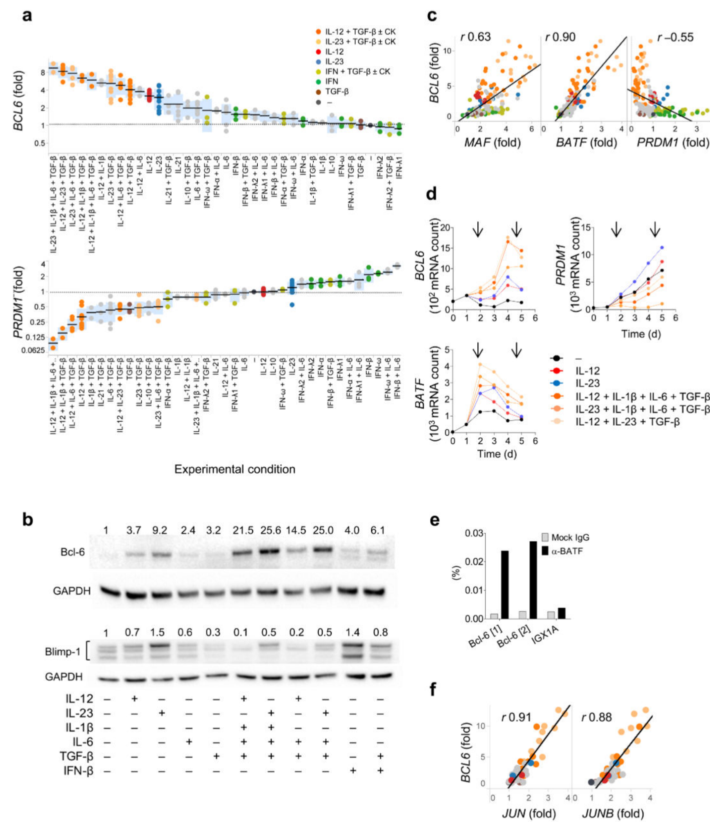

Fig. 2. TGF–β+IL-12 and TGF–β+IL-23 promote the induction of T_{FH} transcriptional signature

(a) Ranking of the differentiation conditions inducing *BCL6* and suppressing *PRDM1* by human adult blood naïve T_H cells cultured as in Fig. 1a. The data were normalized to the values in the culture with no cytokines in each experiment. Each dot represents a result from 11 sets of 4 d culture experiments. Black bars show the mean value and blue boxes indicate ± 1 s.d. **(b)** Immunoblot of Bcl-6 and Blimp-1 with naïve T_H cells cultured with the indicated cytokines for 3 days. The band density after normalization with GAPDH is shown in number. Note that human Blimp-1 has three isoforms. A representative out of 3 independent experiments. **(c)** Correlation between *BCL6* and *MAF*, *BATF*, and *PRDM1* expressed by human naïve T_H cells cultured as in **(a)**. Pearson R values are indicated. P values were all <0.0001. **(d)** Kinetics of *BCL6*, *PRDM1*, and *BATF* expression by naïve T_H cells cultured with cytokines for 1–4 days as in **(a)**. Arrows indicate days when cytokines (CK) were added. **(e)** The chromatin fraction of human naïve T_H cells cultured with IL-23+IL-6+IL-1β+TGF–β for 3 days were immunoprecipitated with anti-BATF or Rabbit IgG, followed by quantitative PCR analysis of the two BATF binding sites within *BCL6* promoter. Results were normalized to input. *IGX1A* region represents a negative control. A representative out of 5 independent experiments. **(e)** Correlation between *BCL6* and *JUN* and *JUNB* expressed by cord blood naïve T_H cells cultured as in **(a)**. Pearson R values are indicated. P values were both <0.0001.

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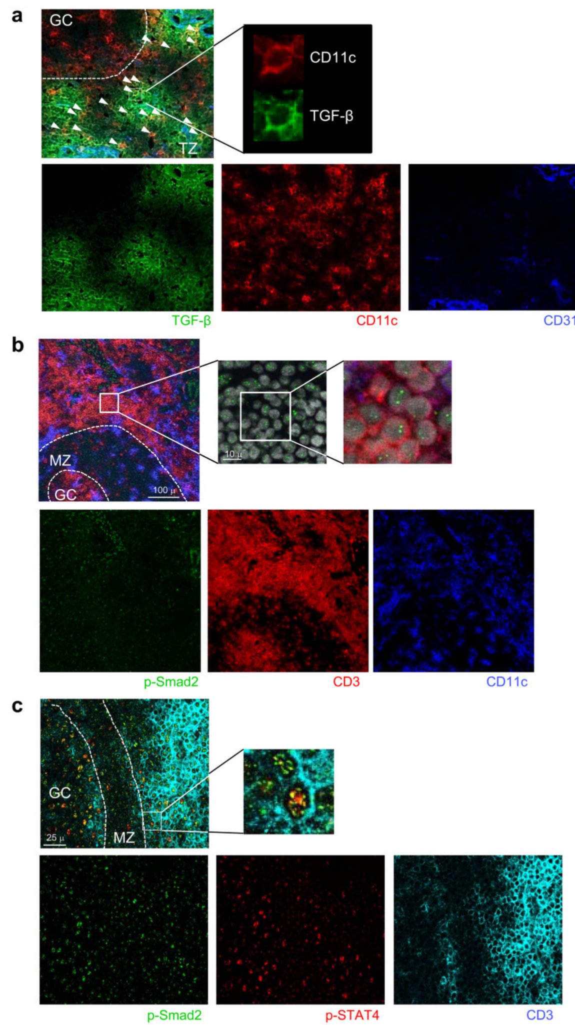

Fig. 3. T_H cells receive TGF–β and IL-12 signals in the T cell zone

(a) Tonsil sections were stained for the expression of TGF–β (indicated by green), CD11c (red), and CD31 (blue) and analyzed with fluorescent microscopy GC: germinal center. TZ: T cell zone. In the top left panel, small arrows indicate cells positive for both CD11c and TGF–β. A representative cell is shown in the top right panel. A representative out of 3 independent experiments. **(b)** Tonsil sections were stained for p-Smad2 (indicated by green), CD11c (blue), and CD3 (red) and analyzed with confocal microscopy. The top right panels show the localization of p-Smad2 in DAPI^{+} nuclei in T cells in the close proximity of GCs. A representative out of 3 independent experiments. **(c)** Tonsil sections were stained for p-Smad2 (indicated by green), p-STAT4 (red), and CD3 (light blue) and analyzed with confocal microscopy. The top right panels show T cells positive for both p-Smad2 and p-STAT4 in the close proximity of GCs. A representative out of 3 independent experiments.

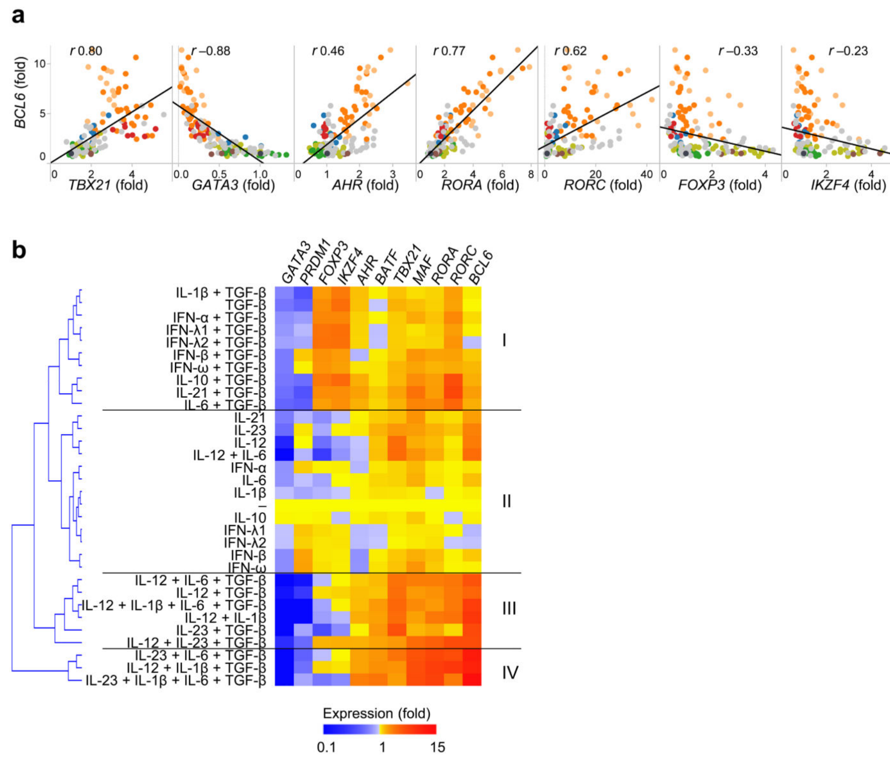

Fig. 4. TGF–β+IL-12 and TGF–β+IL-23 induce both T_{FH} and T_H17 transcriptional signatures

(a) Correlation between *BCL6* and the indicated transcription factors expressed by human adult blood naïve T_H cells cultured for 3 days with different cytokines as in Fig. 2a. Pearson R values are indicated. P values were all <0.0001, except for *IKZF4* (*P*=0.0003). **(b)** An unsupervised clustering of the culture conditions according to the expression of transcription factors by adult naïve TH cells cultured with various cytokines for 3 days as shown in Fig. 2a. For the analysis, the data were first normalized to the values in the culture with no cytokines in each experiment, and the mean values from 11 sets of experiments were determined.

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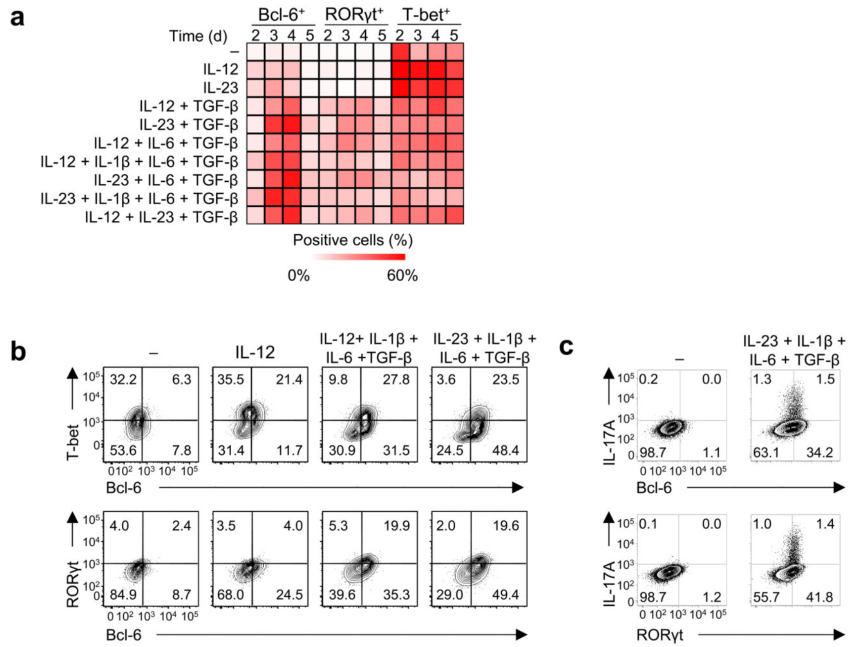

Fig. 5. RORγt is expressed by CXCR5+ Bcl-6+ T_H cells generated with TGF–β+IL-12 and TGF–β +IL-23

(a) The expression of Bcl-6, RORγt, and T-bet by human adult blood naïve T_H cells cultured with the indicated cytokines for 1–4 days (cytokines were added on day 1 of culture, so culture days 2–5) was analyzed by flow cytometry. Percentage of positive cells within the activated ($FSC^{hi}SSC^{hi}$) T_H cells is indicated in a heatmap. A representative of 4 independent experiments. **(b)** The expression of Bcl-6, RORγt, and T-bet by $CXCR5^+$ TH cells generated by culturing naïve T_H cells with no cytokine, IL-12, IL-12+IL-1β+IL-6+TGF-β and IL-23+IL-1β+IL-6+TGF-β for 3 days as in **(a).** A representative of 4 independent experiments. **(c)** The expression of IL-17A, Bcl-6, and RORγt by activated ($FSC^{hi}SSC^{hi}$) cord blood naïve T_H cells cultured for 3 days with no cytokines or IL-23+IL-1β+IL-6+TGF-β. A representative of 3 independent experiments.

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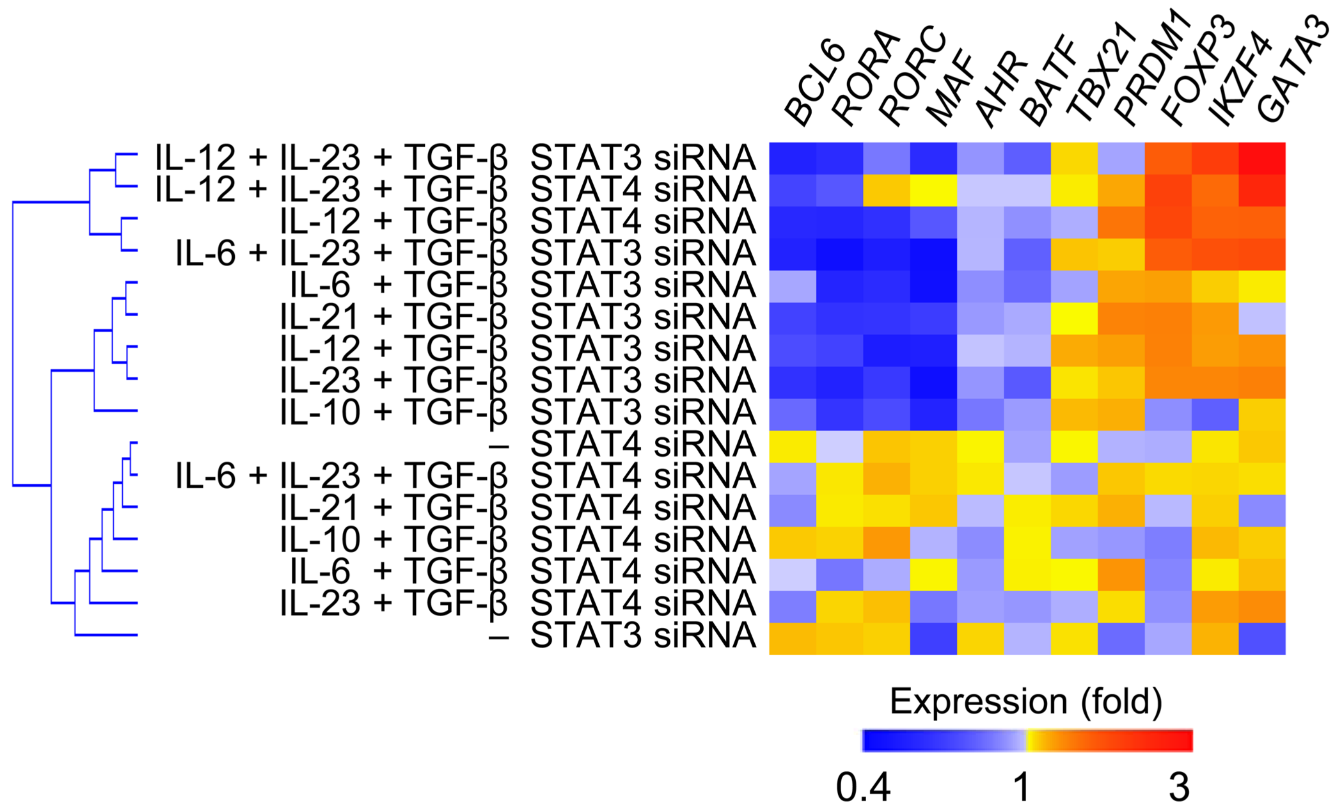

Fig. 6. Both STAT3 and STAT4 cooperate with TGF–β in the induction of T_{FH} and T_H17 transcriptional signatures

The expression of transcription factors by human naïve T_H cells transfected with STAT3 or STAT4 siRNA followed by 2 d culture with combinations of TGF–β with each of the following: IL-12, IL-23, IL-6, IL-12+IL-23, IL-6+IL-23, IL-10 and IL-21. For the analysis, the transcript values in STAT siRNA-transfected T_H cells were first normalized to those in scrambled siRNA-transfected T_H cells cultured in the same cytokine conditions in each experiment, and the mean values from 3 experiments were determined.

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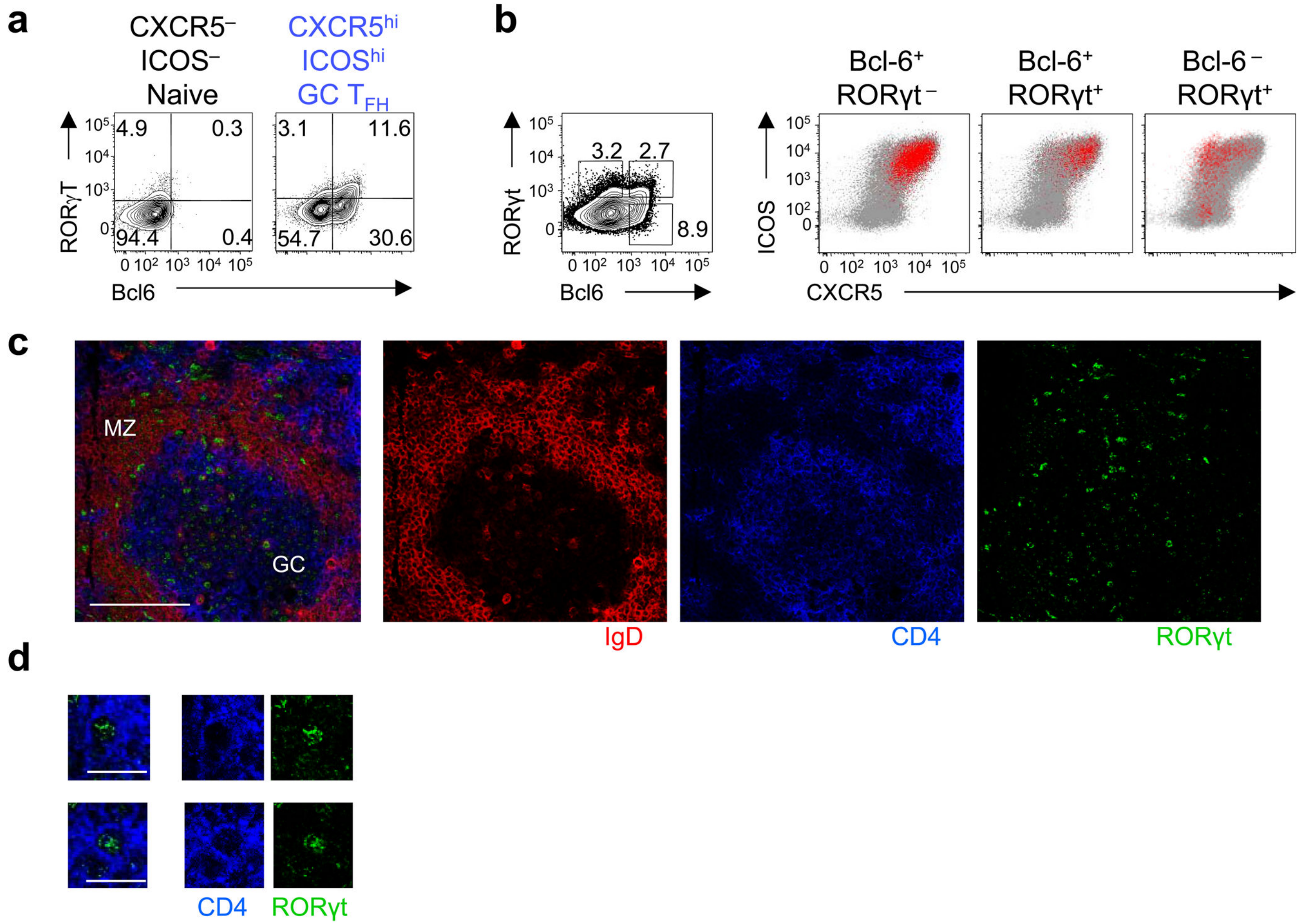

Fig. 7. A fraction of tonsillar T_{FH} cells co-express RORγt

(a) Bcl-6 and RORγt expression by ex vivo tonsillar GC T_{FH} cells and naïve T_H cells was analyzed by flow cytometry. A representative of 4 independent experiments. **(b)** Expression of CXCR5 and ICOS by ex vivo tonsillar Bcl-6$^+$RORγt$^-$,Bcl-6$^+$RORγt$^+$ and Bcl-6$^-$RORγt$^+$ T_H cells (defined as shown in the left panel). The red dots indicate the expression of CXCR5 and ICOS by the cells with the indicated transcription factor expression pattern. The global tonsillar T_H cells are indicated by gray dots. A representative out of 5 independent experiments. **(c)** Human tonsil sections were stained for IgD (indicated by red), CD4 (indicated by blue), and RORγt (indicated by green), and analyzed by confocal microscopy. GC: germinal center. MZ: mantle zone. Representative RORγt$^+$ T_H cells in GCs are shown in panel (**d**).

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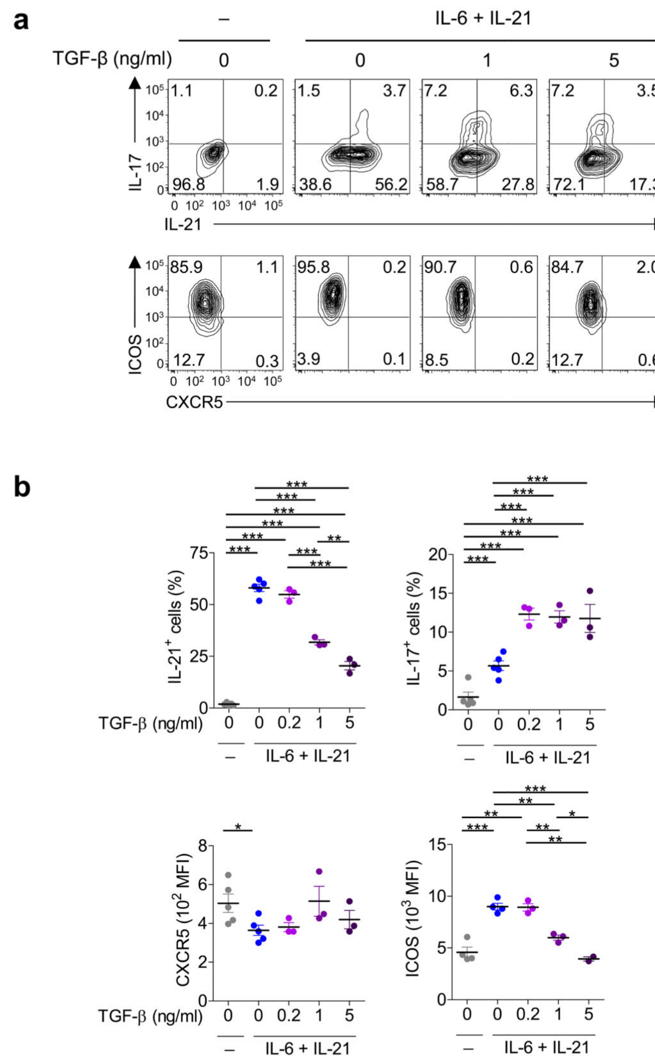

Fig. 8. TGF–β inhibits IL-21 and ICOS expression by mouse T_H cells

(a) Expression of CXCR5, ICOS, and IL-21 by activated ($FSC^{hi}SSC^{hi}$) mouse naïve T_H cells following 4 d stimulation with CD3-CD28 mAbs in the presence of titrated amounts of TGF-β plus IL-6+IL-21 during the last 3 days. A representative of 4 independent experiments. Statistical analysis of results from 5 sets of experiments is shown in panel **(b)**. Two-sided *t*-test: p-values * <0.05, ** <0.01, and *** <0.001.