T CELLS

IL-12 drives the differentiation of human T follicular regulatory cells

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T follicular regulatory (T_{fr}) cells can counteract the B cell helper activity of T follicular helper (T_{FH}) cells and hinder the production of antibodies against self-antigens or allergens. A mechanistic understanding of the cytokines initiating the differentiation of human regulatory T (T_{reg}) cells into T_{fr} cells is still missing. Herein, we report that low doses of the pro-T_{FH} cytokine interleukin-12 (IL-12) drive the induction of a T_{fr} cell program on activated human T_{reg} cells while also preserving their regulatory function. Mechanistically, we found that IL-12 led to STAT4 (signal transducer and activator of transcription 4) phosphorylation and binding to IL-12–driven follicular signature genes. Patients with inborn errors of immunity in the *IL12RB1* gene presented with a strong decrease in circulating T_{fr} cells and produced higher levels of anti-actin autoantibodies in vivo. Overall, this study unveils IL-12 as an inducer of T_{fr} cell differentiation in vivo and provides an approach for the in vitro generation of human T_{fr}-like cells.

INTRODUCTION

Most B cell responses to pathogens and vaccines are tightly regulated by T follicular helper (T_{FH}) cells, a specialized subset of CD4 T cells that orchestrate the differentiation of high-affinity long-lived plasma cells and memory B cells (*1*, *2*). When not properly controlled, T_{FH} cells may be associated with the unfavorable production of antibodies directed against self-antigens or allergens (*3*, *4*). The B cell helper activity of T_{FH} cells can be counteracted by T follicular regulatory (T_{fr}) cells (*5*, *6*), a type of regulatory T (T_{reg}) cell localized in B cell follicles. In both mice and humans, T_{fr} cells are considered to be important in suppressing autoantibody generation and antibody responses to allergens (*3*, *4*, *7*, *8*). Hence, uncovering the signals that promote the differentiation of T_{fr} cells has great potential for facilitating the development of new therapeutic approaches.

The biology of T_{fr} cells has predominantly been explored in mouse models. Most murine T_{fr} cells originate from naturally occurring T_{reg} cells upon antigen-driven activation and retain many key features of T_{reg} cells, including the expression of canonical T_{reg} markers such as forkhead box protein 3 (FOXP3), Helios, cytotoxic T-lymphocyte associated protein 4 (CTLA-4), and CD25 (*9*–*11*). In parallel, murine T_{fr} cells also acquire components of the T_{FH} cell transcriptional program, including *CXCR5, BCL6, PDCD1* (gene encoding for the programmed cell death protein 1, PD-1), and the inducible T cell costimulator (ICOS) (*9*–*11*). Human T_{fr} cells coexpress most of the T_{reg} and T_{FH} cell signature markers described in mice (*12*–*15*); however, they display a wider spectrum of PD-1, which might reflect their differentiation status (*12*, *13*).

Studies conducted in animal models revealed that T_{FH} and T_{fr} cell differentiation appears to be dictated by at least partially overlapping signals. As T_{FH} cells, T_{fr} cells differentiate via a multistep multifactorial process initiated upon priming of naive T_{reg} cells by dendritic cells (DCs) (*5*, *16*). The differentiation of both T_{fr} and T_{FH} cells requires T cell receptor (TCR) stimulation (signal 1) and costimulatory signaling, including CD28 and ICOS (signal 2), and depends on Bcl6 expression (*2*, *10*, *11*, *17*). Several cytokines (signal 3) have been reported to influence T_{FH} cell differentiation and function (*1*, *2*); however, very little is known about the capacity of cytokines to shape the biology of T_{fr} cells. Whereas interleukin-2 (IL-2) has been described to play an inhibitory effect on the differentiation of both T_{FH} and T_{fr} cells (*18*–*20*), the T_{FH} cell–inducing cytokine interleukin-21 (IL-21) was shown to inhibit mouse T_{fr} cell differentiation (*21*). Hence, not all cytokines promoting T_{FH} cell differentiation can, similarly, induce the generation of T_{fr} cells from naive T_{reg} cells. The lack of understanding of how cytokines regulate T_{fr} cell biology is a major knowledge gap that has prevented the development of an in vitro approach for the differentiation of human T_{fr} cells. Multiple cytokines have been described as inducers of human T_{FH} cells (*22*–*25*). Interleukin-12 (IL-12) was first reported to influence the induction of T_{FH} cell signature molecules and IL-21 production in human CD4 T cells (*22*, *23*, *25*). Subsequently, we and others have shown that activin A and transforming growth factor–β (TGF-β) were intrinsically capable of promoting the acquisition of a T_{FH} gene program, and their activity was potentiated by the presence of the IL-12 family members IL-12 and interleukin-23 (IL-23) (*24*, *26*).

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Herein, we show that, like for T_{FH} cells, the IL-12/IL-12R pathway and IL-23, to a lesser extent, are major drivers for the conversion of human naive T_{reg} cells into T_{fr}-like cells. Conversely, activin A and TGF-β had a limited intrinsic ability to drive phenotypical changes in in vitro activated T_{reg} cells. Mechanistically, we showed that signal transducer and activator of transcription 4 (STAT4) could bind several follicular signature genes driven by IL-12, suggesting a direct regulation, in both T_{reg} and conventional CD4 T cells, with a few relevant exceptions. Individuals with mutations on the *IL12RB1* gene displayed strongly reduced frequencies of circulating T_{fr} (cT_{fr}) cells in peripheral blood. Together, our study uncovers a previously unappreciated role of IL-12 as a promoter of the human T_{fr} cell program and provides an approach for the in vitro generation of human T_{fr}-like cells.

RESULTS

Bona fide human T_{fr} cells express low levels of T_{FH} signature markers

During their maturation, human and murine T_{fr} and T_{FH} cells express increasing levels of CXCR5 and PD-1 and can be initially found in follicular mantles (T_{fr} and T_{FH}; $CXCR5^{lo\text{-}int}PD\text{-}1^{lo/int}$) and then in germinal centers (GCs) at later maturation stages (GC-T_{fr} and GC-T_{FH}; $CXCR5^{hi}PD\text{-}1^{hi}$) (*5*, *6*, *12*–*14*, *27*). Of note, most T_{fr} cells detected in human lymph nodes are located in follicular mantles or T-B borders, and only a few T_{fr} cells can reach the GCs (*13*). In follicular mantles, T_{fr} cells express PD-1 and CXCR5 at lower levels in comparison with T_{FH} cells (*13*). To confirm and extend these findings, we directly evaluated the expression of CXCR5, PD-1, and other canonical markers expressed by T_{FH} and T_{reg} cells on activated, total antigen-experienced T_{fr} cells from human pediatric tonsils (fig. S1A). T_{fr} cells were distinguished by a higher expression of PD-1 than naive and antigen-experienced non-T_{fr} cells. In line with previous data (*13*, *14*), total T_{fr} cells presented a lower expression of CXCR5 and PD-1 in comparison with total T_{FH} cells (Fig. 1A and figs. S1, A and B). On the basis of their intermediate CXCR5 and PD-1 expression level, most of the cells in the total T_{fr} gate (>90%) displayed a $CXCR5^{int}PD\text{-}1^{lo\text{-}int}$ T_{fr} phenotype in contrast with T_{FH} cells, which predominantly presented a $CXCR5^{hi}PD\text{-}1^{hi}$ GC phenotype (GC-T_{FH}; >60%) (Fig. 1, B to D). As a result of this distribution, the ratio of T_{fr} cells to T_{FH} cells was higher than the GC-T_{fr}/GC-T_{FH} ratio (fig. S1C).

After stratifying total follicular populations into T_{fr}, GC-T_{fr}, T_{FH}, and GC-T_{FH} cells, we analyzed additional signature markers (Fig. 1, E to G). BCL6, the lineage-defining transcription factor of T_{FH} and T_{fr} cells (*2*, *10*, *11*), was more highly expressed by GC-T_{FH} and GC-T_{fr} cells in comparison with their respective non-GC counterpart (Fig. 1E). T_{fr} cells expressed lower levels of CXCR5, PD-1, and BCL6 in comparison with T_{FH} cells (Fig. 1, E to G). ICOS was present at high levels in all follicular populations, although it was slightly more highly expressed by T_{fr} and GC-T_{fr} cells compared with their T_{FH} counterparts (Fig. 1H). As expected, the T_{reg} signature markers Helios and CTLA-4 were increased in expression in both T_{fr} and GC-T_{fr} cells compared with their nonregulatory counterparts (Fig. 1, I and J). These data demonstrate that most T_{fr} cells in tonsillar tissues acquire a less mature phenotype (suggestive of follicular mantle localization) and that GC-T_{fr} cells are considerably less numerous than GC-T_{FH} cells.

IL-12 induces the differentiation of human T_{fr}-like cells

Because T_{FH} and T_{fr} cells can share similar requirements for their differentiation (*5*, *6*), we proposed that selected cytokines can drive the differentiation of both cell types. IL-12 has been previously described as an inducer of human T_{FH} cell differentiation and function (*22*, *23*, *25*). IL-12 uses a heterodimeric receptor complex for signaling composed of IL-12Rβ2 and IL-12Rβ1 (which can also form IL-23 receptor complex with IL-23Rα) (*28*). To explore whether IL-12 has the potential to promote the differentiation of naive T_{reg} cells into T_{fr} cells, we evaluated the expression of IL-12Rβ1 on conventional CD4 T cells and T_{reg} cell populations from tonsils (figs. S1A and S2A). Naive conventional CD4 T cells and T_{reg} cells did not express IL-12Rβ1 (Fig. 2, A and B). However, the expression of IL-12Rβ1 was acquired by antigen-experienced conventional $CXCR3^+$ CD4 T cells, which are T helper 1 (T_H1) polarized (*29*), and less so by $CXCR3^-$ CD4 T cells (Fig. 2, A and B). Of note, most $CD45RO^+$ T_{reg} cells were IL-12Rβ1$^+$, regardless of their CXCR3 positivity (Fig. 2, A and B). When $CD45RO^+$ T_{reg} cells were further classified into T_{fr} and non-T_{fr} cells (fig. S1A), the expression of IL-12Rβ1 and the proportion of positive cells for this receptor were similar across most populations (fig. S2, B and C). However, the frequency of IL-12Rβ1$^+$ cells was diminished in GC-T_{fr} cells. IL-12Rβ1$^+$ and IL-12Rβ1$^-$ T_{fr} and GC-T_{fr} cells expressed markers that might indicate a diverse positioning and function of these cells (fig. S2D). Specifically, IL-12Rβ1$^+$ GC-T_{fr} cells displayed a higher expression of CCR7 combined with lower CXCR4 and CD69 expression in comparison with their IL-12Rβ1$^-$ counterpart (fig. S2D), suggesting that they might be located closer to the T-B borders and further away from GC dark zones (*30*–*32*). Moreover, IL-12Rβ1$^+$ GC-T_{fr} cells (and to a lesser extent T_{fr} cells) showed higher CD38 compared with their IL-12Rβ1$^-$ counterparts, which could indicate, along with the lower CD69 expression, a lower regulatory activity based on previous findings (*13*, *33*).

To evaluate whether naive T_{reg} cells start expressing IL-12Rβ1 after activation, we followed the induction of IL-12Rβ1 in naive CD4 T cell populations stimulated with anti-CD3/CD28 beads over time. A progressive increase in IL-12Rβ1 expression in both conventional CD4 T cells and T_{reg} cells was evident after 12 hours and reached statistical significance 48 hours after stimulation (Fig. 2C). Moreover, we found that both conventional CD4 T cells and, to a lesser extent, T_{reg} cells became capable of responding to IL-12 upon activation, as measured by the capacity of these cells to rapidly phosphorylate STAT4 after exposure to IL-12 (Fig. 2D). These data show that T_{reg} cells express IL-12Rβ1 after activation and can respond to IL-12 in vitro by phosphorylating STAT4.

Activin A, a pleiotropic cytokine of the TGF-β superfamily (*34*, *35*), is a key inducer of human T_{FH} cell differentiation in vitro (*24*). In contrast with IL-12R, activin A type I (ACVR1B/ALK4) and type II (ACVR2A or ACVR2B) receptors, which are both required for activin A signal transduction (*35*), are constitutively expressed in conventional naive CD4 T cells (*24*). Similarly, naive T_{reg} cells from both cord blood (Fig. 2E) and peripheral blood (fig. S2E) expressed *ACVR1B* and *ACVR2A* and, like conventional CD4 T cells (*24*), down-regulated activin A type I and type II receptors after stimulation (Fig. 2E). We observed that phosphorylation of SMAD2/3, the canonical signaling pathway triggered by activin A (*34*), occurred in naive T_{reg} cells as early as 15 min after incubation with activin A. By contrast, galunisertib, an inhibitor of ALK5 tested in clinical trials

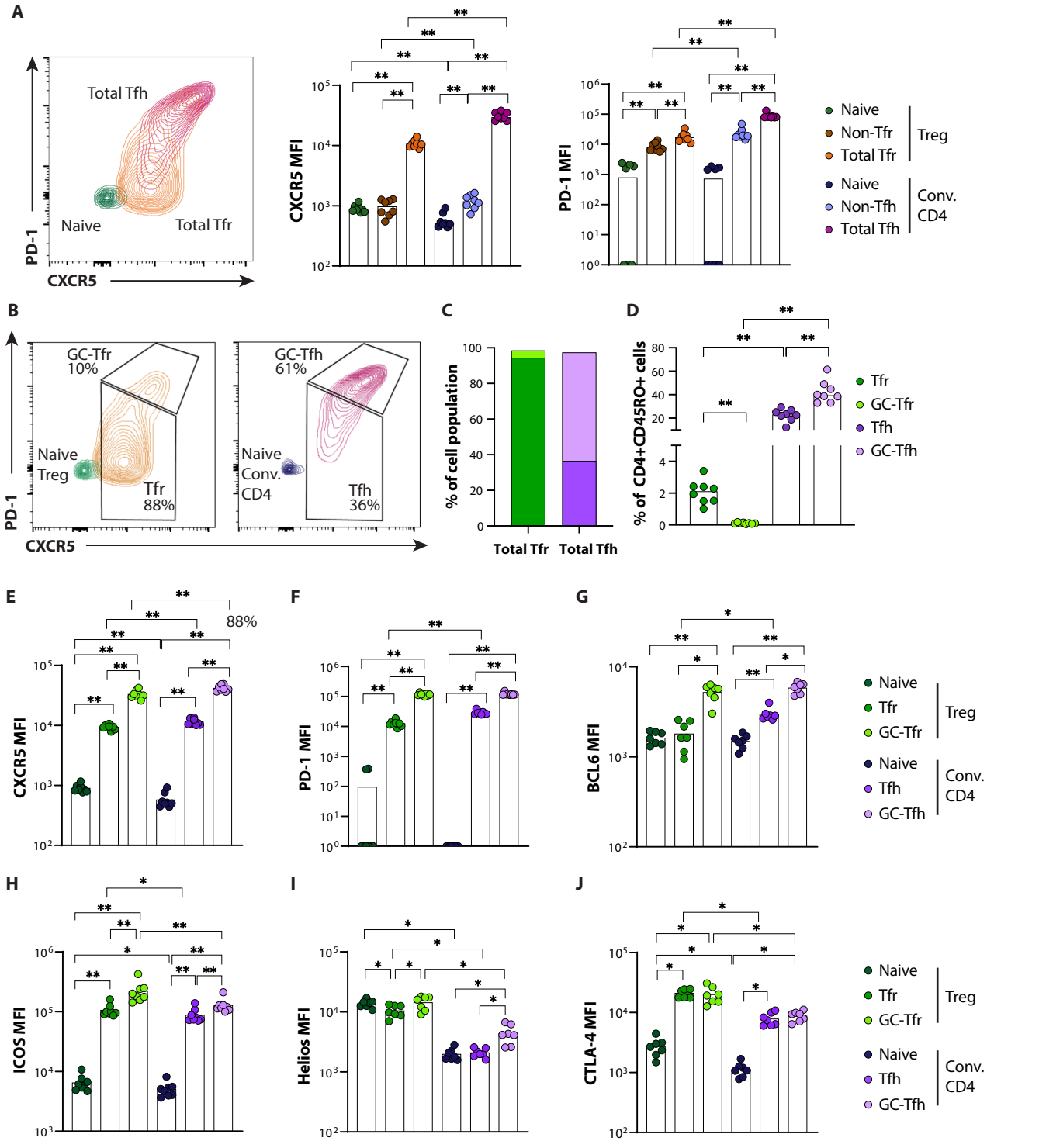

Fig. 1. Human T_{fr} and T_{FH} cells adopt distinct mantle and GC phenotypes. Flow cytometry of human tonsil cells. (**A**) Left: representative contour plot overlay showing CXCR5 and PD-1 expression in naive CD4 T cells (dark green), total T_{fr} cell (orange), and total T_{FH} (dark pink) cells. Center and right: mean fluorescence intensity (MFI) of CXCR5 (center) and PD-1 (right) in T_{reg} and conventional (conv.) CD4 T cell populations; $n = 8$ donors. (**B**) Left: representative contour plots showing T_{fr} and GC-T_{fr} cell gating (pregated on total T_{fr}, orange). Naive T_{reg} cells (green) are shown as a control. Right: T_{FH} and GC-T_{FH} cell gating (pregated on total T_{FH}; dark pink). Naive conventional CD4 T cells (dark blue) are shown as a control. (**C**) Frequencies of T_{fr} (dark green) and GC-T_{fr} (light green) within total T_{fr} cells; frequency of T_{FH} (dark purple) and GC-T_{FH} (light purple) within total T_{FH} cells. (**D**) Frequencies of the populations defined in (C) within CD45RO$^+$ CD4 T cells; $n = 8$ donors. (**E** to **H**) MFI of the T_{FH} signature markers CXCR5 (E), PD-1 (F), BCL6 (G), and ICOS (H) in T_{reg} and conventional CD4 T cell populations; $n = 7$ donors. (**I** and **J**) MFI of the T_{reg} cell signature markers Helios (I) and CTLA-4 (J) in T_{reg} and conventional CD4 T cell populations; $n = 7$ donors. [(A) and (D) to (J)] Individual samples and mean are shown. Wilcoxon test, $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; and $^{****}P < 0.0001$.

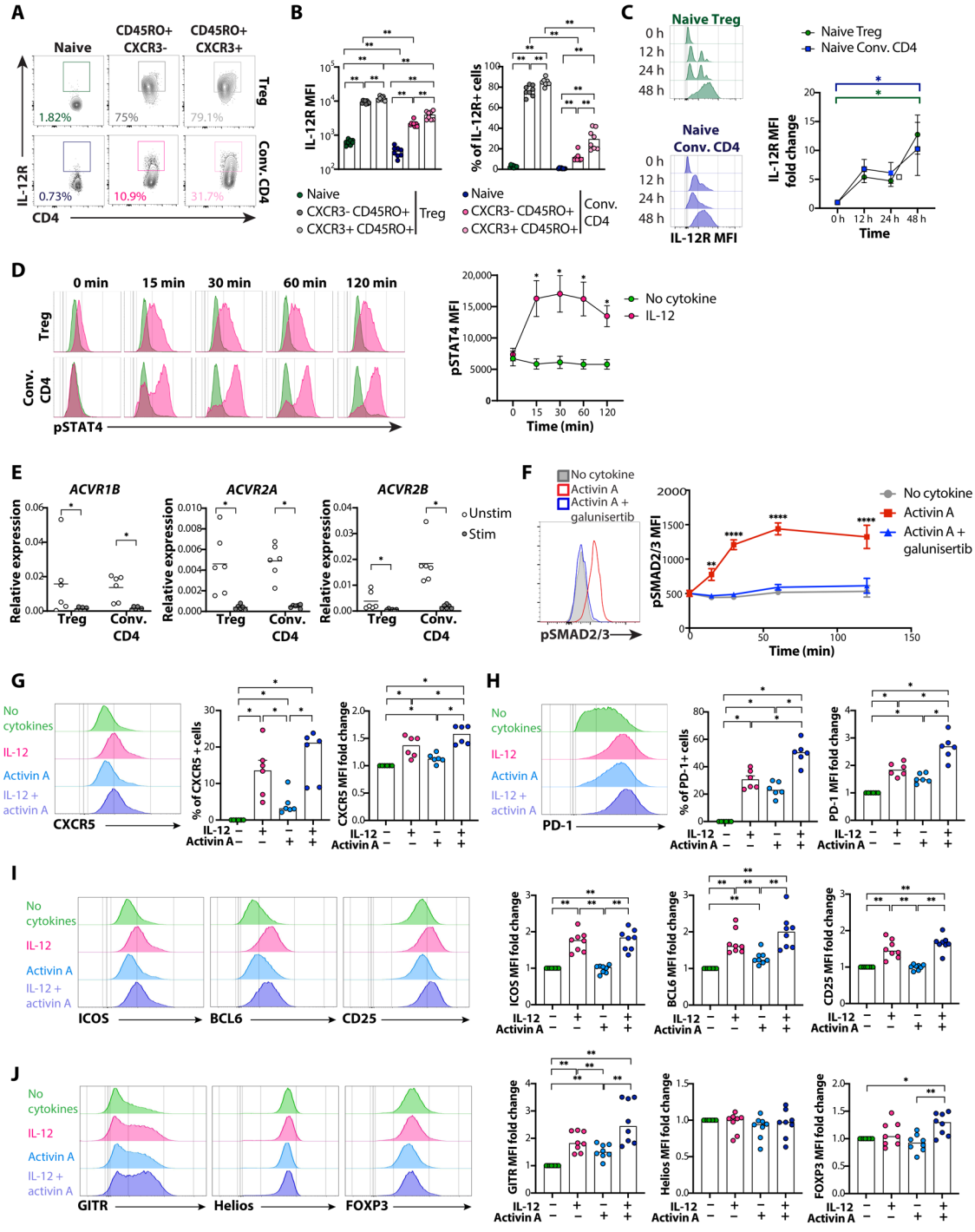

Fig. 2. T_{reg} cells express IL-12Rβ1 and acquire expression of T_{fr} signature markers in response to IL-12. (**A**) Representative plots of IL-12Rβ1 expression (IL-12R) in naive ($CD45RO^-$) and antigen-experienced ($CD45RO^+$) T_{reg} and conventional CD4 T cell (gating strategy in fig. S2A). (**B**) MFI and frequency of $IL\text{-}12R^+$ cells in populations described in (A); $n = 8$ donors. (**C**) Representative histograms and kinetics of IL-12R (MFI) expression in naive T_{reg} cells ($n = 4$ donors) and naive conventional CD4 T cells ($n = 5$ donors) from cord blood after in vitro activation. (**D**) Left: representative histograms of phosphorylated STAT4 (pSTAT4) in naive T_{reg} cells and naive conventional CD4 T cells activated and cultured with (dark pink) and without (green) IL-12. Right: kinetic of pSTAT4 (MFI) in T_{reg} cells as shown in (D). $n = 5$ donors. (**E**) Gene expression of activin A receptors in naive (Unstim) T_{reg} cells and naive conventional CD4 T cells or activated (Stim) cells. The comparisons were performed across the study groups at every time point; $n = 6$ donors. (**F**) Left: representative histograms of SMAD2/3 phosphorylation (pSMAD2/3) in naive T_{reg} cells after in vitro culture with activin A, with and without galunisertib. Right: kinetic of pSMAD2/3 (MFI) as shown in (F); unstimulated cells in gray. The comparisons were performed across the study groups at every time point; $n = 3$ donors. (**G** to **J**) Activated naive T_{reg} cells from cord blood were differentiated with or without IL-12 and/or activin A. Representative histograms, frequency, and MFI fold change over the "no cytokines" condition for CXCR5 (G) and PD-1 (H), $n = 6$ donors; ICOS, BCL6, and CD25 (I), $n = 8$ donors; and GITR, Helios, and FOXP3 (J), $n = 8$ donors. [(B), (E), and (G) to (J)] Individual samples and mean are shown. Wilcoxon test. [(C), (D), and (F)] Data are shown as mean and SEM. Two-way ANOVA and Šídák posttest. [(B) to (J)] $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; and $^{****}P < 0.0001$.

that can also inhibit ALK4 (*36*), potently inhibited activin A–mediated phosphorylation of SMAD2/3 in T_{reg} cells (Fig. 2F). Overall, these results show that naive T_{reg} cells express activin A receptors and can respond to activin A in vitro by activating the SMAD2/3 signaling pathway.

We next assessed whether IL-12 and activin A could favor the in vitro differentiation of naive T_{reg} cells into T_{fr}-like cells. To this aim, we activated naive T_{reg} cells with anti-CD3/CD28 beads in the presence of IL-12 and activin A, independently or in combination. Because high levels of IL-12 in culture systems have been associated with loss of regulatory function by T_{reg} cells (*37*), we used a low IL-12 concentration (5 ng/ml). The addition of IL-12 led to an increase in the expression of CXCR5 and PD-1 as well as in the frequency of $CXCR5^+$ and $PD\text{-}1^+$ cells in cord blood T_{reg} cells (Fig. 2, G and H). Conversely, activin A only weakly induced the expression of CXCR5 and PD-1 and moderately amplified the IL-12–mediated induction of these molecules (Fig. 2, G and H). Similar results were observed with naive T_{reg} cells from peripheral blood (fig. S3A). Of note, IL-12 also enhanced the expression of the canonical T_{fr} cell markers ICOS and BCL6 and the T_{reg}-associated molecules CD25 and GITR (glucocorticoid-induced tumor necrosis factor receptor-related protein) while not affecting the expression of Helios and FOXP3 (Fig. 2, I and J). The addition of activin A to IL-12 only modestly increased the expression of GITR, whereas ICOS, BCL6, CD25, Helios, and FOXP3 expression levels were minimally or not influenced by activin A (Fig. 2, I and J). In vitro cultures with naive T_{reg} cells from peripheral blood yielded similar trends (fig. S3B).

Next, we asked whether higher concentrations of IL-12 (25 and 50 ng/ml) could elicit a more pronounced induction of the follicular markers tested above. We did not see a dose-dependent effect, and medium/high concentrations of IL-12 resulted in a similar induction of CXCR5, PD-1, and ICOS as the low concentration (fig. S3C). BCL6 was significantly induced only by low/medium IL-12 concentrations but not by the highest dose. In addition, all IL-12 concentrations similarly increased CD25 expression, and no dose had an impact on HELIOS and FOXP3 expression (fig. S3D). These data suggest that minimal concentrations of IL-12 are sufficient for the conversion of naive activated T_{reg} cells into T_{fr}-like cells. Last, we also observed that the induction of the follicular markers CXCR5 and PD-1 on activated T_{reg} cells cultured in vitro with IL-12 was lower than that induced on conventional CD4 T cells from the same donor (fig. S3E). Overall, these data demonstrated that IL-12 can imprint several facets of the follicular cell program on human activated T_{reg} cells while leaving the expression of certain regulatory components intact. Conversely, we did not observe a major role for activin A in human T_{fr} cell differentiation.

IL-23 is less potent than IL-12 in promoting a T_{fr}-like phenotype

Like IL-12, the IL-12 family member IL-23 contains the p40 subunit and requires a receptor complex including IL-12Rβ1 to signal (*28*). Hence, we investigated whether IL-23, alone or in combination with a broader panel of cytokines known to modulate T_{FH} cell differentiation (*24*, *26*), could also drive a T_{fr}-like phenotype in activated human T_{reg} cells. We found that IL-23 promoted similar levels of CXCR5 and BCL6 as IL-12, whereas PD-1 and ICOS were more efficiently induced by IL-12 than by IL-23 (Fig. 3, A to D). Of note, unlike IL-12, IL-23 failed to induce CD25 expression and down-regulated Helios and FOXP3 compared with the condition without exogenous cytokines (Fig. 3, E to G). Furthermore, the addition of activin A to IL-23 did not enhance IL-23's capacity to induce CXCR5, PD-1, ICOS, and BCL6, nor did it rescue Helios and FOXP3 expression.

Among the other cytokines tested, interleukin-10 (IL-10) displayed a minor effect on most markers analyzed. Conversely, TGF-β alone was able to elicit PD-1 and BCL6 expression (Fig. 3, B and D) and increase PD-1 (Fig. 3B), CD25, and FOXP3 expression (Fig. 3G) when combined with IL-12. We did not, however, find a synergistic effect between TGF-β and IL-12 or IL-23 as previously observed in conventional CD4 T cells (*26*). Together, these data show that IL-23 induced canonical follicular markers on activated T_{reg} cells but less efficiently than IL-12 and did not preserve the expression of T_{reg} signature genes. These data suggest that IL-12 may play a stronger role in human T_{fr} cell biology than IL-23.

T_{fr}-like cells induced by low IL-12 doses retain their regulatory function

To assess whether IL-12 hinders the regulatory function of T_{reg} cells, we evaluated the function of the in vitro activated T_{reg} cells precultured with a low IL-12 concentration. First, we evaluated expression of CTLA-4, an established mediator of T_{fr} cell function (*38*, *39*). We observed a mild but significant increase in the expression of CTLA-4 when activated T_{reg} cells were cultured with IL-12 alone or with activin A (Fig. 4A). As a second approach, we used a classical suppression assay, where the suppression of effector CD4 T cell (T_{eff}) ($CD4^+CD25^-$ cells) proliferation by T_{reg} cells was measured. All activated T_{reg} cells, precultured with or without cytokines, were able to suppress the proliferation of T_{eff} cells and the induction of the activation marker CD25 on these cells (Fig. 4, B to F). Although the suppressive capacity of the cells precultured with IL-12 appeared slightly reduced compared with T_{reg} cells precultured without cytokines, exposure to a low dose of IL-12, overall, allowed T_{reg} cells to retain their suppressive function (Fig. 4, B to F). Moreover, we found that the in vitro cultured T_{reg} cells that gained CXCR5 expression had a comparable suppressive activity to that of the $CXCR5^-$ T_{regs} from the same culture, regardless of whether they were preexposed to IL-12 (Fig. 4D).

Third, we determined the suppressive function of in vitro differentiated T_{reg} cells in the setting of a T-B coculture assay where T_{fr} cells suppress T_{FH} cell–mediated plasmablast differentiation and antibody production by memory B cells (*40*). The incubation of tonsillar GC-T_{FH} cells and memory B cells with the superantigen staphylococcal enterotoxin B (SEB) resulted in a robust differentiation of plasma cells and elevated production of antibodies (Fig. 4, G to I). These responses were suppressed in the presence of T_{reg} cells previously differentiated in vitro with IL-12, activin A, or a combination of the two. Of note, the T_{reg} cells precultured with a low IL-12 concentration preserved their capacity to inhibit T_{FH}-mediated plasmablast differentiation as well as the secretion of immunoglobulin G (IgG) and immunoglobulin A (IgA) (Fig. 4, G to I). Similar results were observed using T_{FH} cells instead of GC-T_{FH} cells as B cell helpers (fig. S4, A to C).

Subsequently, we asked whether higher concentrations of IL-12 dampened the suppressive activity of the in vitro differentiated T_{fr}-like cells. We found that, whereas a low IL-12 concentration promoted up-regulation of CTLA-4, the high IL-12 dose used in our study did not (Fig. 4A and fig. S4D). Furthermore, in line with published data (*37*), there was a pronounced loss in the capacity to

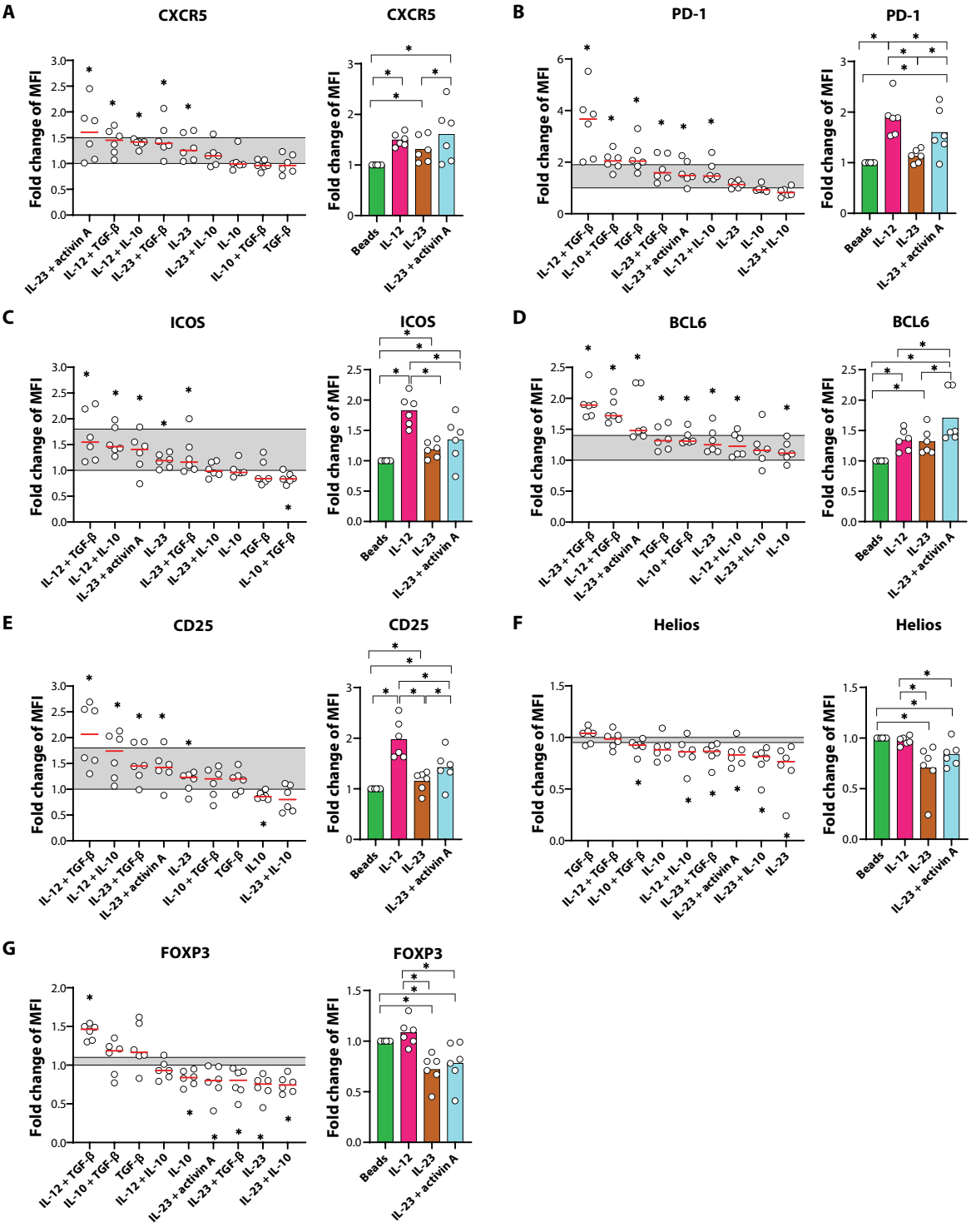

Fig. 3. IL-23 is less potent than IL-12 in promoting a T_{fr}-like phenotype. (**A** to **G**) Naive T_{reg} cells from cord blood or peripheral blood were cultured with anti-CD3/CD28 beads for 5 days, with or without IL-12, IL-23, TGF-β, and/or IL-10 alone or in combination. Activin A was also included only in combination with other cytokines. [(A) to (D)] Quantification of MFI fold change over the "no cytokines" condition for CXCR5 (A), PD-1 (B), ICOS (C), and BCL6 (D); $n = 6$ donors. [(E) to (G)] Quantification of MFI fold change for the T_{reg} signature molecules CD25 (E), Helios (F), and FOXP3 (G); $n = 6$ donors. [(A) to (G)] All individual samples and mean (red) are shown; the gray area separates the mean of the samples from the "no cytokines" (bottom line) and the "IL-12" treatment (top line) groups. Statistical differences refer to comparison with the "no cytokines" group. Wilcoxon test, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; and $^{****}P < 0.0001$.

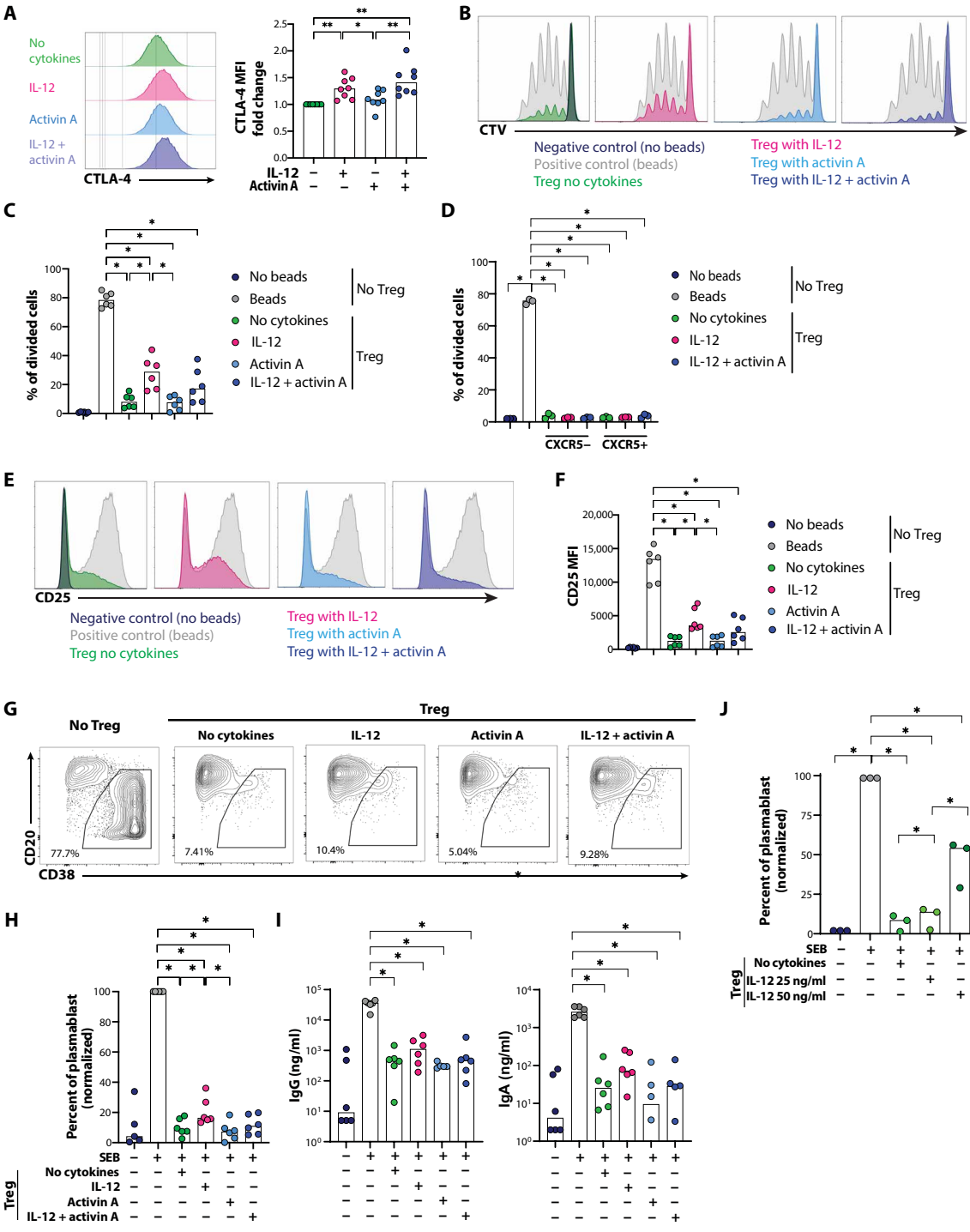

Fig. 4. T_{reg} cell suppressive function is preserved after in vitro culture with IL-12. (**A**) Representative histograms and MFI fold change over the "no cytokines" condition for CTLA-4 in naive T_{reg} cells from cord blood cultured with or without IL-12 (5 ng/ml) and/or activin A (50 ng/ml); $n = 8$ donors. (**B**) Representative histograms showing the proliferation of T_{eff} cells cultured with anti-CD3/CD28 beads alone (gray) or with in vitro differentiated allogenic T_{reg} cells. CTV, CellTrace Violet. (**C** and **D**) Quantification of T_{eff} proliferation as described in (B), in the presence of total allogenic T_{reg} ($n = 6$ donors) (C) or with $CXCR5^-$ and $CXCR5^+$ T_{reg} populations ($n = 3$ donors) (D) from in vitro cultures. (**E**) Representative histograms of CD25 in T_{eff} cells cultured with anti-CD3/CD28 beads alone (gray) or with in vitro differentiated total allogenic T_{reg} cells. (**F**) Quantification of CD25 MFI in T_{eff} cells as described in (E). $n = 6$ donors. (**G** and **H**) Representative contour plots (G) and quantification (H) of plasmablasts at day 7 of the coculture of memory B cells with autologous GC-T_{FH} cells in the presence or absence of in vitro differentiated allogenic T_{reg} cells. $n = 6$ donors. (**I**) Levels of IgG (left) and IgA (right) in the supernatants of the cocultures shown in [(G) and (H)]. $n = 6$ donors. [(A) to (I)] The in vitro allogeneic T_{reg} cells were differentiated with IL-12 (5 ng/ml) and/or activin A (50 ng/ml). (**J**) Quantification of plasmablasts at day 7 of the coculture of memory B cells with autologous GC-T_{FH} cells, with in vitro allogeneic T_{reg} cells differentiated with different concentrations of IL-12. $n = 3$ donors. [(A), (C), (D), (F), and (H) to (J)] Individual samples and mean are shown. Wilcoxon test, $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; and $^{****}P < 0.0001$.

suppress T_{FH} help to B cells within the population of T_{reg} cells that were precultured with the high IL-12 concentration (Fig. 4J).

Last, we tested whether a low dose of IL-23, which led to a significant loss in Helios and FOXP3 expression (Fig. 3), resulted in a loss of suppressive function. Although IL-23 failed to induce a significant up-regulation of CTLA-4 (fig. S4E), in the setting of a T-B coculture assay, T_{reg} cells precultured with a low dose of IL-23 maintained the ability to suppress T_{FH} help to B cells in vitro (fig. S4F). In sum, while a high concentration of IL-12 can affect the suppressive ability of human T_{regs}, low IL-12 dose allows the acquisition of follicular markers by T_{reg} cells without markedly hindering their regulatory activity. Similarly, a low dose of IL-23 did not affect the suppressive function of human T_{reg} cells in vitro.

T_{fr} and IL-12–driven T_{fr}-like cells display T_H1 and follicular gene signatures

IL-12 is a key regulator of the T_H1 gene program in human and murine CD4 T cells (*29*, *41*, *42*). Hence, if IL-12 physiologically plays a role in the in vivo differentiation of human T_{fr} cells, then this could be reflected by the presence of a T_H1 gene signature in bona fide T_{fr} cells. To verify this hypothesis, we performed RNA sequencing (RNA-seq) on T_{fr} cells from tonsils (gating strategy, fig. S5A; purity after sorting >92%, fig. S5B) and compared their transcriptome with that of naive CXCR5$^-$ T_{reg} cells. As anticipated, and in line with previous studies (*14*, *15*), bona fide T_{fr} cells simultaneously displayed a pronounced expression of T_{reg} (*43*) and T_{FH} canonical genes (*24*) (Fig. 5A). Furthermore, elevated levels of T_H1-associated gene transcripts, including *TBX21*, *CXCR3*, and *IL12RB2* (*44*), were observed in T_{fr} cells in comparison with naive T_{reg} cells (Fig. 5A). Validation of selected markers that were not previously tested by flow cytometry corroborated a heightened expression of the T_{reg} cell–associated molecule LAG3 (lymphocyte-activation gene 3), the T_{FH} transcriptional regulator MAF, and the T_H1 transcription factor T-bet on bona fide T_{fr} cell populations (T_{fr} and GC-T_{fr} cells) (Fig. 5B). A Gene Set Enrichment Analysis (GSEA) approach confirmed that tonsillar bona fide T_{fr} cells were overall enriched in genes associated with activated T_{reg} cells, bona fide T_{FH} cells, and T_H1 cells (Fig. 5C) (*24*, *43*, *44*).

We next performed a transcriptomic analysis of activated T_{reg} cells cultured in vitro with IL-12 to determine the enrichment in genes associated with activated T_{reg} cells, bona fide T_{FH} cells, and T_H1 cells (Fig. 5D). We observed an increased expression of T_{reg} (*CTLA-4*, *LAG3*, *FURIN*, and *HAVCR2*), T_{FH} (e.g., *MAF*, *ICOS*, *PDCD1*, and *CXCR5*), and T_H1 (*TBX21*, *IL-12RB2*, *CXCR3*, and *IL23R*)–associated genes in T_{reg} cells cultured with IL-12 compared with activated T_{reg} cells cultured without exogenous cytokines (Fig. 5D). We further confirmed by quantitative polymerase chain reaction (qPCR) that IL-12 treatment up-regulated the T_H1 signature gene *TBX21* (Fig. 5E). Moreover, higher levels of *LAG3* and *FURIN* transcripts were detected with IL-12 treatment (alone or in combination with activin A) (Fig. 5E). Despite their enrichment in T_H1 signature genes, we only detected a very marginal production of interferon-γ (IFN-γ) by T_{reg} cells polarized in vitro with a low dose of IL-12 compared with conventional CD4 T cells (fig. S5C). We also confirmed a higher *MAF* expression on T_{reg} cells cultured in vitro with IL-12 (Fig. 5E), along with the previously shown capacity of these cells to express higher levels of BCL6, CXCR5, PD-1, and ICOS (Fig. 2). Last, among all genes differentially expressed in T_{reg} cells cultured with IL-12 in comparison to T_{reg} cultured without cytokines, one-third of those genes were up-regulated or down-regulated by bona fide T_{fr} cells in comparison with naive T_{reg} cells (fig. S5D), showing a broader enrichment in bona fide T_{fr}-associated genes. Together, these data support a model in which IL-12 acts as a major stimulus for the acquisition of the T_{fr} gene program by activated naive T_{reg} cells. Moreover, our data revealed that both bona fide T_{fr} cells, like in vitro differentiated T_{fr}-like cells, express molecules associated with T_H1 polarization.

STAT4 binds follicular genes in T_{reg} and conventional CD4 T cells upon IL-12 exposure

To gain mechanistic insights into how IL-12 regulates the differentiation of human T_{fr}-like cells, we asked whether STAT4 directly binds IL-12–induced follicular signature genes in T_{reg} cells cultured with IL-12. Toward this goal, we optimized a CUT&RUN approach to assess the STAT4 genome occupancy. In parallel, we conducted a similar study on conventional naive CD4 T cells to determine whether IL-12 exposure leads to overlapping STAT4 occupancy in T_{reg} and conventional CD4 T cells. Our assay was specific, as shown by the elevated number and frequency of peaks measured in both T_{reg} and conventional CD4 T cells cultured with IL-12 in comparison with cells cultured without exogenous IL-12 (Fig. 6A, fig. S6, A and B, and data file S1). In both cell types, STAT4 binding was abundant in intronic and intergenic regions and slightly enriched at the transcription start site when compared with the background genomic distribution (fig. S6A). Of note, in T_{reg} cells cultured with IL-12, we observed elevated STAT4 binding in all IL-12–induced follicular genes (Figs. 2 and 5), including *CXCR5*, *BCL6*, *MAF*, *PDCD1* , *ICOS*, *IL2RA* (encoding CD25), and *TNFRSF18* (encoding GITR) (Fig. 6B, fig. S6C, and data file S1). These data suggest that the regulation of such follicular genes in T_{reg} cells might be directly mediated by STAT4.

Next, we evaluated similarities and differences in STAT4 binding between naive T_{reg} cells and conventional CD4 T cells. In both cell types, IL-12 strongly stimulated the binding of STAT4 at target genes, with a slightly stronger effect for T_{reg} cells, both at the level of peak number and total STAT4-bound sequence space (fig. S6B). Although most target regions were bound by STAT4 on both T_{reg} and conventional CD4 T cells (13,792), a few were selectively bound on T_{reg} (284) or conventional CD4 T (54) cells (Fig. 6A and fig. S6, C and D). When coupling the STAT4 occupancy data with the transcriptomic datasets from T_{reg} and conventional CD4 T cells cultured with IL-12 or no exogenous cytokines (*24*), we observed that most genes up-regulated by IL-12 were bound by STAT4 in both T_{reg} and conventional CD4 T cells (Fig. 6C). Down-regulated genes were bound by STAT4 to a lesser extent.

We next asked whether differential STAT4 binding in conventional CD4 versus T_{reg} cells in response to IL-12 could lead to differential gene expression. Seven genes that were induced by IL-12 and preferentially expressed in T_{regs} contained T_{reg}-specific STAT4 peaks (*ANKRD22*, *CTLA-4*, *FN1*, *GCNT1*, *SETBP1*, *TMEM169*, and *TSPAN13*, shown in red, Fig. 6D), whereas 14 genes induced by IL-12 and preferentially expressed in conventional CD4 T cells contained STAT4 peaks specific to this cell type (*BBS12*, *CASK*, *CDO1*, *CDYL2*, *CHST11*, *IFNG*, *IFNG-AS1*, *IL21*, *IL21-AS1*, *IL26*, *LOC105370413*, *MDM1*, *MXI1*, and *SLC12A8*; shown in blue, Fig. 6D). A complete list of the genes differentially bound by STAT4 in T_{reg} or conventional CD4 T cells is provided in data file S2. We found heightened binding of STAT4 in proximity to the *CTLA-4* gene in

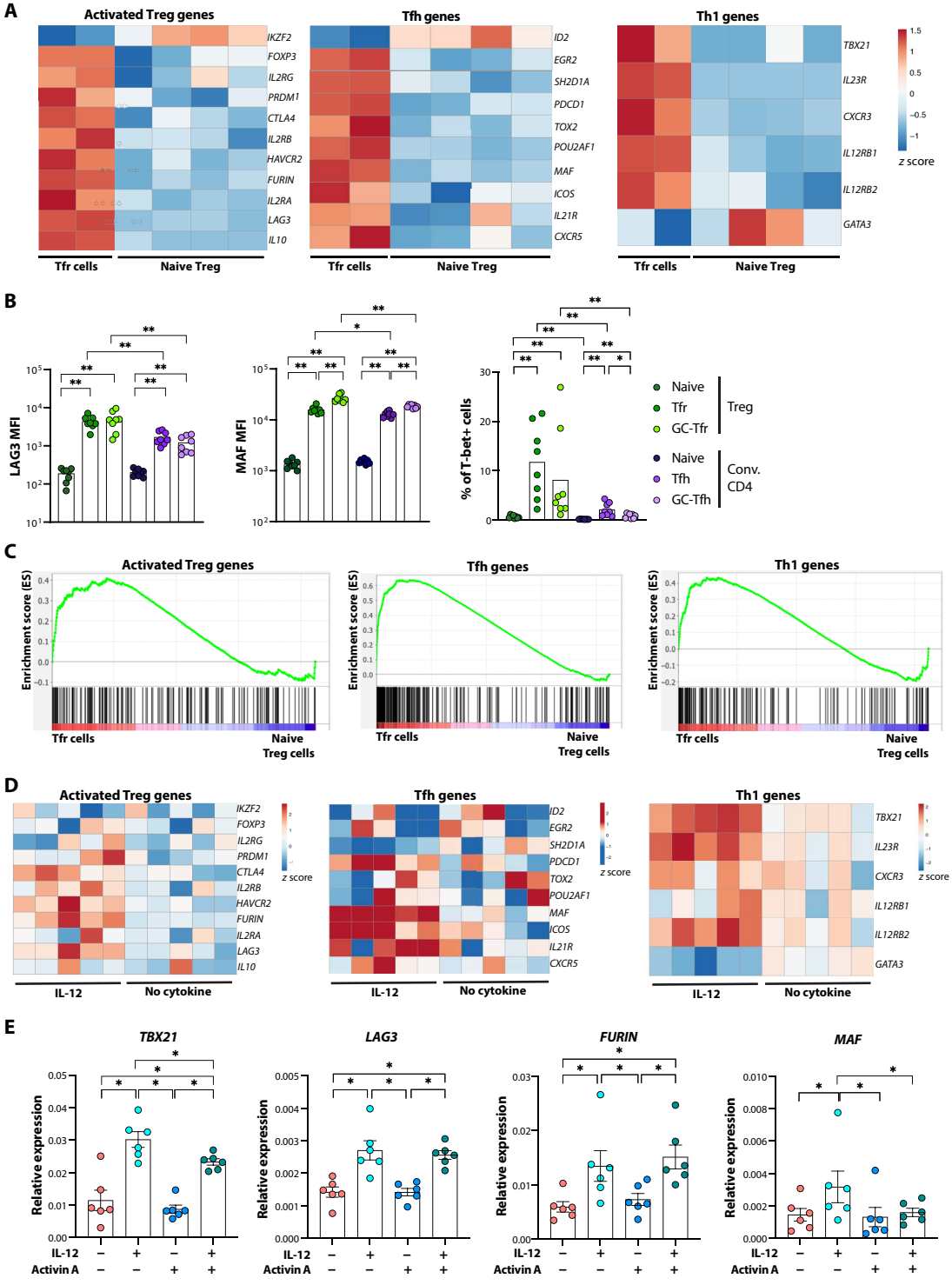

Fig. 5. T_H1 and follicular signature genes are expressed by T_{fr} cells and T_{reg} cells cultured with IL-12. (**A**) Heatmap analysis of RNA-seq data of bona fide tonsillar T_{fr} cells and naive T_{reg} cells from cord blood, showing the expression of activated T_{reg} (left), T_{FH} (center), and T_H1 (right) signature genes. All genes displayed in (A) are significantly up-regulated or down-regulated in T_{fr} in comparison with naive T_{reg} cells (P value < 0.05), except GATA3, for which $P = 0.20$. (**B**) Validation of LAG3 (left), MAF (center), and T-bet (right) expression by flow cytometry. (**C**) GSEA showing the enrichment of activated T_{reg} (left), T_{FH} (center), and T_H1 (right) signature genes in tonsillar bona fide T_{fr} cells versus naive T_{reg} cells. (**D**) Heatmap analysis of RNA-seq data from cord blood naive T_{reg} cells cultured with IL-12 or without exogenous cytokines (no cytokines), showing the expression of T_{reg} (left), T_{FH} (center), and T_H1 (right) signature genes. All genes were differentially expressed in cells cultured with IL-12 in comparison with the "no cytokines" condition, with a P value < 0.05 except for *IKZF2*, *FOXP3*, *IL2RG*, *PRDM1*, *EGR2*, *TOX2*, *POU2AF1*, *IL21R*, and *IL12RB1*. (**E**) From left to right: validation of *TBX21*, *LAG3*, *FURIN*, and *MAF* gene expression by qPCR. [(B) and (E)] Individual samples and mean are shown. Wilcoxon test, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; and ****$P < 0.0001$.

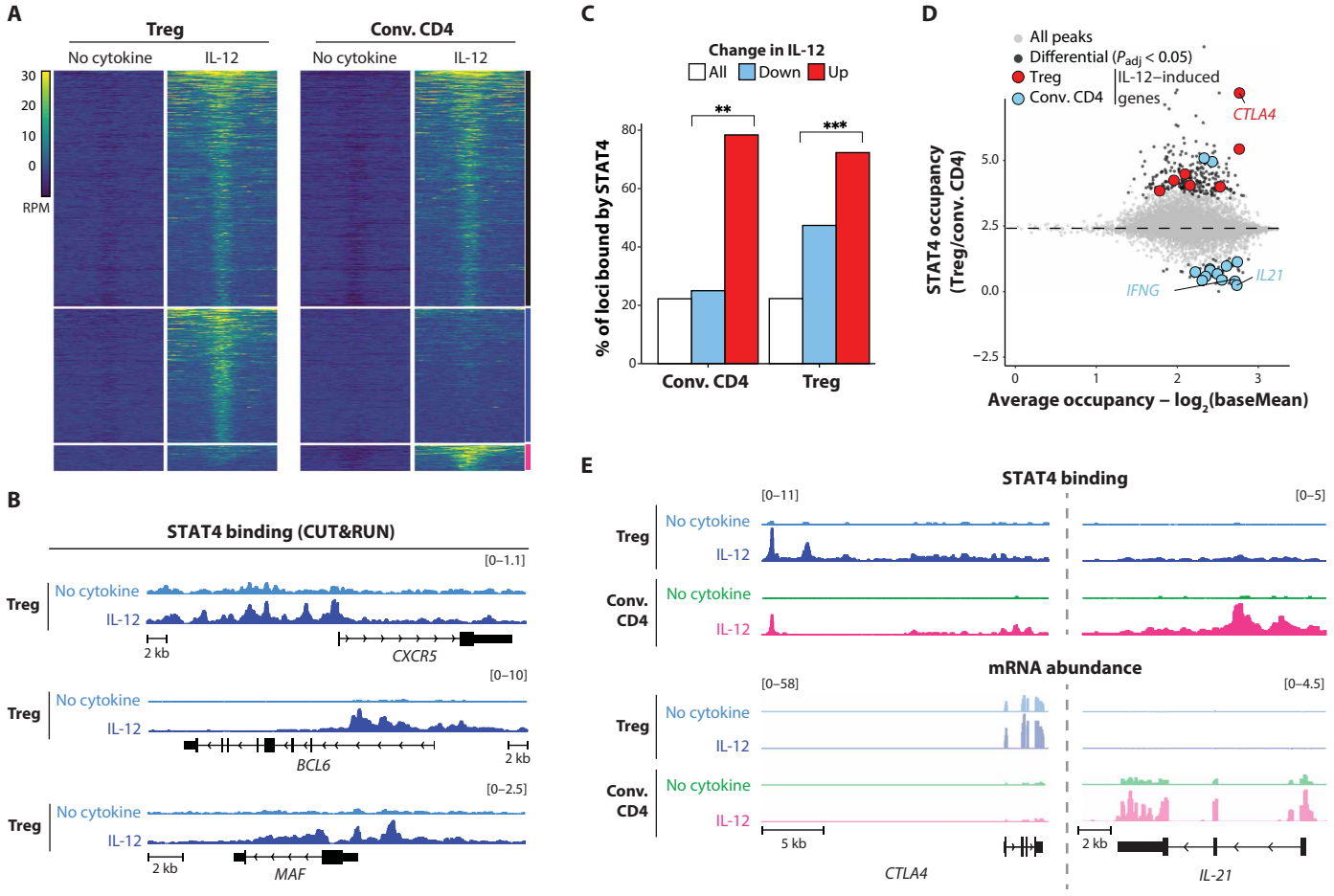

Fig. 6. STAT4 binds to follicular genes in both T_{reg} and conventional CD4 T cells. (**A**) Library-size normalized for STAT4 CUT&RUN after subtracting signal from control IgG reactions. Regions shown are bound by STAT4 after IL-12 stimulation of conventional CD4 T cells (bottom right: pink bar, $n = 54$ genes), T_{reg} cells (middle right: blue bar, $n = 284$ genes), and in both cell types (top right: black bar, $n = 13{,}792$ genes, of which only 500 are shown for clarity). Windows shown span ±2.5 kb from the peak center. Data from three biological replicates. RPM, reads per million. (**B**) Genome browser images for STAT4 CUT&RUN signal at three example genes up-regulated by IL-12 in T_{reg} cells. The *y* axis represents reads per million. Scale bars for the *x* axis indicate 10 kb. Tracks show the mean from three biological replicates. (**C**) Bars indicate the portion (%) of each gene category, i.e., genes down-regulated (blue) or up-regulated (red) by IL-12, that also display binding of STAT4 in response to IL-12 at or near the promoter. Fisher's exact tests. (**D**) MA plot for STAT4 binding in IL-12 stimulated cells. The *y* axis represents log-converted differential STAT4 binding in T_{reg} versus conventional CD4 T cells. Peaks were assigned to the nearest gene within 100 kb, and for each gene, only the peak with the greatest fold change was retained. Peaks induced by IL-12 and with biased expression in T_{reg} (red) or conventional CD4 T cells (blue) are highlighted. (**E**) Genome browser images for STAT4 CUT&RUN signal and RNA-seq in T_{regs} or conventional CD4 T cells not treated (no cytokine) or treated with IL-12. The *y* axis represents reads per million. Data from three biological replicates.

T_{reg} cells, which also displayed higher mRNA abundance when cultured with IL-12 (Fig. 6E). This data is consistent with a higher CTLA-4 expression on bona fide T_{fr} cells compared with T_{FH} cells (Fig. 1J). Conversely, an enrichment for STAT4 binding was observed in the *IL21* gene in conventional CD4 T cells but not in T_{reg} cells cultured with IL-12 in comparison to the no cytokine condition, along with a higher *IL21* mRNA abundance in conventional CD4 T cells (Fig. 6E). Another gene that was induced by IL-12 and displayed a stronger STAT4 binding in conventional CD4 was *IFNG* (fig. S6E), supporting the notion that this effector gene is differentially expressed between T_{FH} and T_{fr} cells. Accordingly, IL-12 induced much stronger IFN-γ production in conventional CD4 T cells in comparison with T_{reg} cells (fig. S5C). *CD40LG* displayed a similar trend (fig. S6E). Last, we observed that more STAT4 binding in proximity of the *CXCR5* gene was connected to a trend for a higher *CXCR5* mRNA abundance in conventional CD4 T cells (fig. S6E), which might at least partially explain the higher CXCR5 expression found in bona fide T_{FH} cells compared with T_{fr} cells. In sum, our data suggest that IL-12 directly promotes the induction of a follicular program in T_{reg} cells and, although most of the follicular program is similarly regulated by IL-12/STAT4 in T_{reg} and conventional CD4 cells, there are also cell-intrinsic differences in the regulation of specific genes.

Patients with IL-12Rβ1 deficiency have a reduced frequency of cT_{fr} cells

To further investigate the physiological role of IL-12 in human T_{fr} cell differentiation, we evaluated the abundance of cT_{fr} cells and T_{reg} cells in patients of pediatric age with inborn errors of immunity of the *IL12RB1* gene (IL12R-def; $n = 8$ individuals) and age-matched healthy controls (HCs; $n = 8$ individuals). Demographic information of the study cohort is provided in table S1. Whereas patients

with IL-12Rβ1 deficiency had a normal frequency of naive T_{reg} cells ($CD45RA^+$), we found a decrease in total and antigen-experienced ($CD45RA^-$) T_{reg} cell frequencies compared with the control group (Fig. 7A and fig. S7A). This decrease suggests that IL-12–responsive T_{reg} cell populations, including T_{fr} cells, might be affected in the absence of proper IL-12 signaling. In line with our hypothesis, the population of cT_{fr} cells was strongly decreased in patients with IL-12Rβ1 deficiency (Fig. 7B and fig. S7B). We observed a decrease in both $CD45RA^-$ antigen-experienced cT_{fr} cells and $CD45RA^+$ cT_{fr} cells, which have been hypothesized to reflect intermediate and early T_{fr} differentiation stages, respectively (Fig. 7, C and D) (*5*). Of note, patients with IL-12Rβ1 deficiency displayed a pronounced loss of CXCR5 expression in all CD4 T cells, including circulating T_{FH} cells (Fig. 7C and fig. S7C), in agreement with data from a larger patient group (*23*).

Because T_{fr} cells are important in preventing the production of autoantibodies, we sought to determine whether patients with IL-12Rβ1 deficiency are more likely than healthy individuals to develop autoantibodies. We found a significant increase in the levels of anti-actin IgG in patients with IL-12Rβ1 deficiency (Fig. 7E). Nonetheless, using a custom, multiplexed autoantigen microarray composed of autoantigens targeted in many different connective tissue diseases, we did not identify an overall increase in autoantibody production. The only autoantibodies that were detectable in patients with IL-12Rβ1 deficiency were those targeting the systemic sclerosis U11/U12 ribonucleoprotein (RNP) autoantigen (fig. S7D). Together, these data suggest that IL-12 is an important regulator of T_{fr} cell biology in vivo and that the reduction in cT_{fr} cell frequencies of patients with IL-12Rβ1 deficiency might be associated with the production of autoantibodies against a limited number of autoantigens.

DISCUSSION

Herein, we described an approach for the in vitro differentiation of human T_{fr}-like cells based on exposure to IL-12, simultaneously uncovering an unappreciated role for this cytokine in human T_{fr} cell biology. Most protocols for the in vitro differentiation of T cell subsets rely on the use of specific cytokines. Although T_{fr} cells were described more than a decade ago (*9*–*11*), little is known about the cytokine regulation of T_{fr} cell biology, with our current knowledge limited to two cytokines that were reported to be negative regulators of T_{fr} cells: IL-21 and IL-2 (*18*, *21*). The finding that IL-21, an important cytokine with pro-T_{FH} activity, inhibits instead of promoting T_{fr} cell differentiation highlights that it should not be assumed that all cytokines promoting T_{FH} cell differentiation can also drive T_{fr} cell differentiation. Consistent with this notion, we found that activin A, one of the most potent inducers of the human T_{FH} cell program in vitro (*24*), fails to promote a robust induction of follicular markers in activated T_{reg} cells and does not strongly synergize with other cytokines to drive the differentiation of T_{fr}-like cells. Similarly, TGF-β also fails to synergize with STAT4-inducing cytokines to drive a potent induction of CXCR5 expression, as previously shown for T_{FH}-like cells (*26*). Of note, we show here that IL-12, another well-established inducer of human T_{FH} cell differentiation and function (*22*, *23*, *25*), can induce the expression of key follicular markers on activated naive T_{reg} cells. Although the induction of these follicular markers by IL-12 in T_{reg} cells was reproducible, levels were lower than those induced by IL-12 in conventional naive CD4 T cells. This finding was not unexpected because most T_{fr} cells from human lymphoid organs express considerably lower levels of CXCR5 and PD-1 in comparison with T_{FH} cells, as shown by Sayin *et al.* (*13*) and further corroborated by our data. These data might be explained by more abundant STAT4 binding in certain follicular genes in conventional CD4 T cells compared with T_{reg} cells after exposure to IL-12, as shown in this study for CXCR5, although there are likely additional factors contributing to this phenomenon. The low capacity of T_{fr} cells to express molecules important for GC localization is consistent with their selective localization in follicular mantles and with their ability to express high levels of CD25 (*13*). This enables IL-2 signaling and the expression of BLIMP-1, a transcription factor that potently restrains the follicular program (*18*). T_{fr} cells are characterized by distinct coexpression of BCL6 and BLIMP-1, in contrast with the complete lack of BLIMP-1 expression by T_{FH} cells (*5*, *11*, *14*). Hence, the combination of IL-12 with a low dose of IL-2, which is important for the survival and proliferation of T_{reg} cells in vivo and in vitro, promotes the acquisition of follicular markers at moderate expression levels.

In mice, elevated concentrations of IL-12, which persist in vivo for just a few days in acute infection models, result in IFN-γ production by T_{reg} cells and loss of T_{reg} cell suppressive function (*45*). In humans, T_{reg} cells also produce IFN-γ and lose their suppressive activity in response to high IL-12 doses (*37*). In our study, we exposed naive T_{reg} cells to a low dose of IL-12, in the setting of TCR stimulation and CD28 costimulation. Low-dose IL-12 did not lead to a relevant IFN-γ production by activated T_{reg} cells but rather drove the acquisition of distinctive features of T_{fr} cells. A low-dose IL-12 only caused a minor reduction in the functionality of T_{reg} cells, which overall retained the capacity to suppress effector cell proliferation and T_{FH} cell help to B cells and expressed higher levels of CTLA-4, LAG3, and FURIN. These molecules have been previously associated with T_{fr} cells (*14*, *15*) and the suppression activity of tissue T_{reg} cells (*46*, *47*). Conversely, higher doses of IL-12 led to a substantial loss of regulatory activity without driving greater induction of T_{fr}-associated markers. Our data support a model in which a low IL-12 concentration favors T_{fr} cell differentiation without converting T_{fr} cells into effector cells. By contrast, elevated levels of IL-12, likely present in the early phases of immune responses in vivo, might favor the differentiation of T_{FH} cells while hindering T_{fr} cell suppressive activity.

Our study sheds light on the mechanism behind the capacity of IL-12 to drive a follicular program in activated T_{reg} cells by showing the binding of STAT4, which is rapidly phosphorylated in activated T_{reg} cells in response to IL-12, to T_{fr} signature genes that are upregulated by IL-12, including *CXCR5* and *BCL6*. These data suggest that certain follicular genes are directly regulated by the IL-12/STAT4 pathway, although we cannot rule out that other transcription factors induced by IL-12 could also promote the induction of the follicular markers on T_{fr}-like cells. For instance, *MAF*, which we showed is driven by IL-12 in T_{reg} cells, could contribute to the induction of CXCR5, as shown for naive CD4 T cells (*48*). Another interesting aspect that emerged from our study is the high number of overlapping genes bound by STAT4 in T_{reg} and conventional CD4 T cells upon exposure to IL-12. There were, however, few notable differences, including enriched binding of STAT4 to *CTLA-4* in T_{reg} cells compared with conventional CD4 T cells. This is consistent with the higher expression of CTLA-4 in bona fide tonsillar T_{fr} and CTLA-4 serving as an important molecule for their suppressive activity (*38*, *39*). Conversely, more abundant STAT4 binding to *IL21*

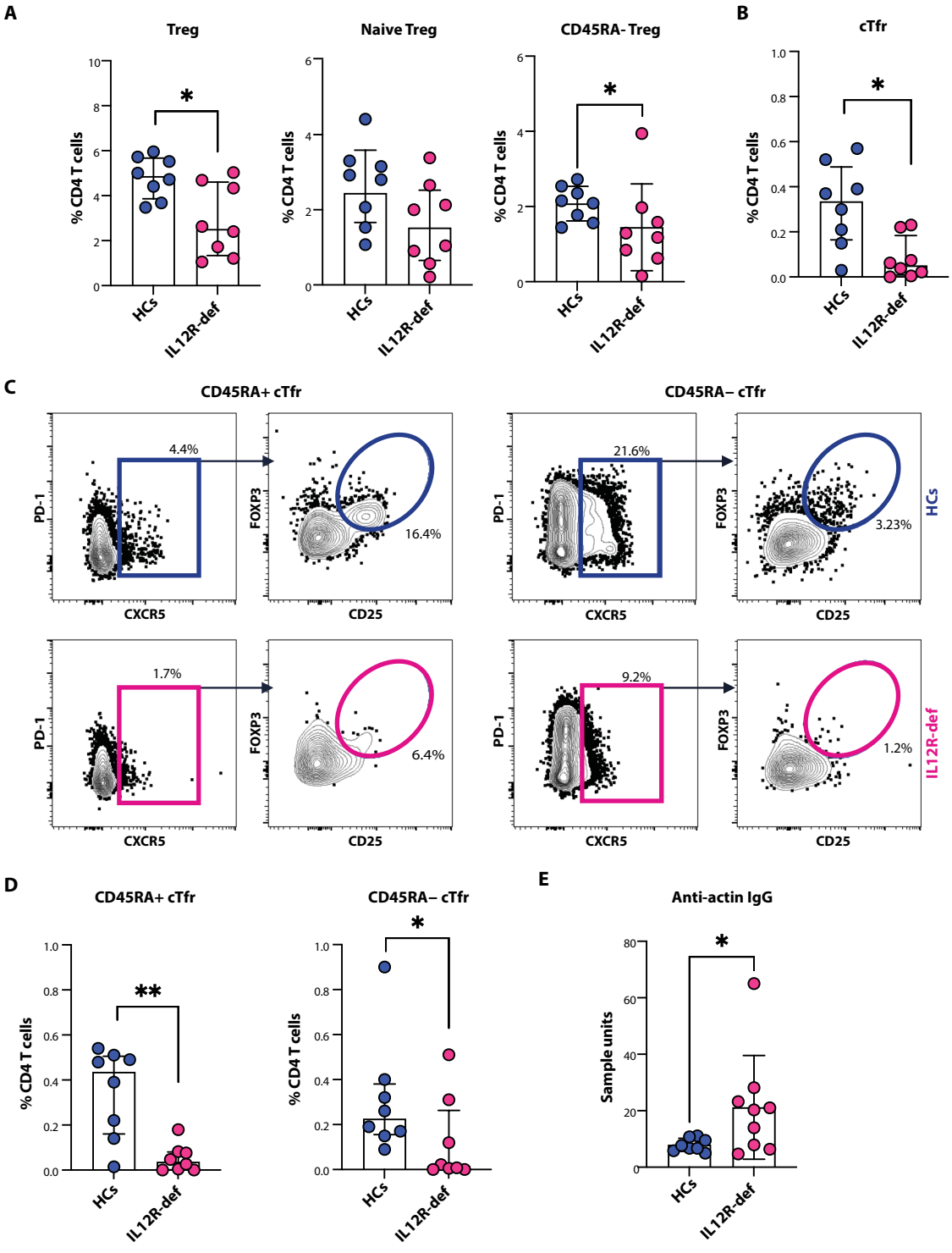

Fig. 7. Patients with IL-12Rβ1 deficiency display low frequencies of cT_{fr} cells. (**A**) Frequencies of (total) T_{reg} cells (left), naive T_{reg} cells (center), and antigen-experienced ($CD45RA^-$) T_{reg} cells (right) from HCs and patients with IL-12Rβ1 deficiency (IL12R-def). (**B**) Frequencies of cT_{fr} cells within the CD4 T cell population in HCs and IL12R-def. (**C**) Representative flow plots showing naive ($CD45RA^+$; left) and antigen-experienced ($CD45RA^-$; right) cT_{fr} cells in HCs (top) and IL12R-def (bottom). Cells were previously gated on live $CD4^+CD45RA^-$ or $CD4^+CD45RA^+$ cells, as detailed in fig. S7A. (**D**) Frequency of naive ($CD45RA^+$; left) and antigen-experienced ($CD45RA^-$; right) cT_{fr} cells within CD4 T cells in HCs and IL12R-def. (**E**) Anti-actin IgG were measured in the serum of HCs and IL-12Rβ1-def; $n = 8$ donors. [(A), (B), (D), and (E)] Individual samples and mean are shown. Mann-Whitney test, $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; and $^{****}P < 0.0001$.

was detected in conventional CD4 T cells, which is an intriguing observation because T_{FH} but not T_{fr} cells can produce IL-21 (*49*). This differential binding could be explained by the presence of additional transcription factors that are exclusively present in T_{fr} or T_{FH} cells (for instance, FOXP3 in T_{reg} cells) and could antagonize STAT4 binding or alter STAT4's binding capacity for selected target genes. Future studies are needed to shed light on this differential regulation by STAT4 in T_{reg} and conventional CD4 T cells.

Building on our in vitro findings, we demonstrated that T_{reg} cells have the potential to respond to IL-12 in vivo because most antigen-experienced T_{reg} cells express IL-12Rβ1. In addition, our transcriptomic analysis of T_{fr} cells from tonsillar tissue indicated that IL-12 signaling might occur in vivo in T_{fr} cells. Bona fide T_{fr} cells were enriched in T_H1 signature genes in comparison with naive T_{reg} cells. Of note, the physiological importance of IL-12 signaling was further corroborated by the observed severe reduction in the frequency of cT_{fr} cells in the peripheral blood of most individuals with inborn errors of immunity affecting IL-12Rβ1. Because IL-12Rβ1 is also required for IL-23 to signal, we cannot exclude that the hindered frequency of cT_{fr} results from a combined inability of the T_{reg} cells in these patients to respond to both IL-12 and IL-23. However, because IL-23's impact on the induction of follicular markers in T_{reg} cells found in our study was milder compared with what we observed in response to IL-12 in vitro, we speculate that IL-12 is a major driver of the cT_{fr} reduction we observed in individuals with IL-12Rβ1 deficiency. It is worth noting that a previous study did not report differences in the frequency of T_{fr} cells in patients with IL-12R deficiency, possibly due to the small number of patients evaluated and a different approach in defining T_{reg} cells that did not include FOXP3 (*50*). Because T_{fr} cells are important in preventing autoantibody production, we also evaluated the production of several autoantibodies in patients with IL-12Rβ1 deficiency. Although we did not observe an overall increase in all antibodies tested, we found heightened levels of autoantibodies against actin and RNP U11/U12 in patients with IL-12Rβ1 deficiency compared with age-matched healthy donors. Anti-actin antibodies have been previously described in patients with IL-12R deficiency (*51*) and are currently used for the diagnosis of autoimmune hepatitis (*52*), whereas anti-U11/U12 RNP antibodies are present in approximately 3% of patients with systemic sclerosis (*53*). The biological impacts of the observed T_{fr} cell decrease, and these autoantibodies in patients with IL-12Rβ1 deficiency remain to be established. Patients with IL-12R deficiency are usually characterized by increased susceptibility to intramacrophagic pathogen infection, and there are only rare reports of autoimmune manifestations in these patients, typically during chronic infections (e.g., salmonellosis) (*54*–*57*). An important factor, however, is that T_{FH} cell help is usually required for autoantibody production, and a severe reduction in T_{FH} cell frequency was reported by this and another study in patients with IL-12Rβ1 deficiency (*23*).

A caveat associated with the study of human T_{fr} cells is the fact that all T_{reg} cells (including non-T_{fr} cells) can suppress T_{FH} help to B cells in vitro (*12*, *13*, *15*). The lack of preferential suppression of humoral responses by T_{fr} cells in vitro suggests that the T_{fr} program does not instruct an additional suppressive function on T_{reg} cells but rather works by conferring T_{reg} cells with the capacity to express molecules (e.g., CXCR5, ICOS, and PD-1) that might facilitate their positioning in vivo at T-B borders or in GCs. This localization requirement is bypassed in vitro, making it challenging to assess whether certain stimuli selectively contribute to the functional properties of T_{fr} cells. Despite these challenges, our study identifies an underappreciated role for IL-12 as a regulator of human T_{fr} cell biology in vitro and in vivo, with important implications for therapeutic approaches seeking to manipulate this cell population.

MATERIALS AND METHODS

Study design

The primary goal of this study was to assess the impact of IL-12 and activin A, two cytokines molding the differentiation and function of T_{FH} cells, on human T_{fr} cells. Additional cytokines were also tested for selected assays among those described below. We first measured, by flow cytometry or qPCR, the expression of the receptors for IL-12 and activin A on various T_{reg} cell populations. Then, we adopted an in vitro experimental approach to evaluate the cytokine effect on the differentiation of human naive T_{reg} cells into T_{fr} cells. We assessed T_{fr} cell differentiation at three different levels: (i) induction of signature markers, (ii) regulation of the transcriptional profile, and (iii) function. Next, we performed a STAT4 occupancy study to determine STAT4 binding to follicular signature genes in response to IL-12 in T_{reg} and conventional CD4 T cells. Last, we defined the frequencies of cT_{fr} cells and the production of autoreactive antibodies in two pediatric patient cohorts: patients with IL-12Rβ1 deficiency and age-matched HCs.

Human samples

Fresh human cord blood samples were obtained from the New York Blood Center (New York, NY), Carolina Cord Blood Bank (Duke University, Durham, NC), and Vitalant Clinical Services (Allendale, NJ). The samples were shipped overnight no longer than 18 hours after their collection. Fresh, deidentified human tonsils were obtained from the Children's Hospital of Philadelphia (CHOP) (Philadelphia, PA). Fresh peripheral blood mononuclear cells (PBMCs) were obtained from the Human Immunology Core (University of Pennsylvania, Philadelphia, PA). Fresh buffy coat samples were obtained from BioIVT (Hicksville, NY) and were shipped overnight. Cryopreserved PBMCs and serum from patients with *IL-12RB1* deficiency, with previously reported (*58*–*60*) and unidentified mutations resulting in a complete IL-12Rβ1 deficiency, were collected at the Necker Hospital, Paris, France (table S1). These patients were recruited by clinical suspicion of Mendelian susceptibility to mycobacterial diseases. A biallelic variant in *IL12RB1* was detected in these patients by whole exome and/or Sanger sequencing. PBMCs from healthy, age-matched (3 to 18 years-old), and sex-matched controls (not ancestry-matched) were collected at CHOP. All the protocols for sample collections were approved by the Institutional Review Board, and informed consent was obtained by the institutions where the samples were collected.

Cell sorting for in vitro differentiation

Total CD4 T cells were enriched from cord blood or peripheral blood using the EasySep Human $CD4^+$ T Cell Isolation Kit (STEMCELL Technologies), following the manufacturer's instructions. Cells were stained with Fixable Viability dye eFluor 780 and fluorescently labeled anti-human monoclonal antibodies (listed in table S2) in fluorescence-activated cell sorting (FACS) buffer [phosphate-buffered saline (PBS) with 2% fetal bovine serum] for 30 min at 4°C. Cells were washed in FACS buffer, collected in tubes with 35-μm nylon mesh caps, and immediately sorted by flow cytometry;

naive T_{reg} cells were gated as live $CD4^+CD45RO^-CD25^{Hi}CD127^{Lo/-}CXCR5^-$ cells; naive conventional CD4 T cells as live $CD4^+CD45RO^-CD25^-CD127^+$. Samples were sorted using a FACSAria Fusion (BD Biosciences) with a 70-μm nozzle, an Aurora CS spectral cell sorter (Fremont, CA) with a 100-μm nozzle, or a FACSMelody (BD Biosciences) with a 100-μm nozzle. The purity of naive T_{reg} cells was subsequently evaluated on the basis of the percentage of $FOXP3^+$ cells and was always ≥95%. Sorted cells were immediately used for in vitro differentiation.

In vitro T_{reg} cell differentiation

Freshly isolated naive T_{reg} cells (7.5×10^4 cells per well) from cord blood or peripheral blood were cultured at 37°C in serum-free AIM-V with 2 μl of Dynabeads Human T-Activator CD3/CD28 and activin A (50 ng/ml), IL-12 (5 ng/ml), recombinant human IL-23 (5 ng/ml), recombinant human TGF-β (2 ng/ml), and/or recombinant human IL-10 (10 ng/ml) (R&D Systems), in the presence of IL-2 (5 ng/ml). In selected experiments, higher concentrations of IL-12 were tested (25 and 50 ng/ml). After 2 days, cells from each condition were split into two wells and cultured with fresh AIM-V medium without further cytokine addition. After 5 total days in culture, flow cytometry or functional tests were performed on the in vitro differentiated cells. Naive conventional CD4 T cells were also cultured with the cytokine combinations above with interleukin-7 (IL-7) (4 ng/ml) instead of IL-2, as previously reported (*24*). No splitting was required for naive conventional CD4 T cells.

T_{FH} cell–B cell–T_{reg} cell coculture

T cell–B cell coculture was previously described in detail (*40*). Tonsillar T_{FH} and B cells were isolated from cryopreserved samples. $CD19^+$ cells were enriched using the EasySep Human CD19 Positive Selection Kit II (STEMCELL Technologies). Memory B cells were sorted by flow cytometry from the $CD19^+$ cells as live $CD3^-CD4^-CD14^-CD19^+IgD^-CD27^+CD38^-$ cells. The negative fraction of the CD19 isolation was used to sort T_{FH} cells as live $CD8^-CD14^-CD19^-CD4^+CD45RA^-CXCR5^+PD1^{-/Lo}$ cells or GC-T_{FH} cells as live $CD8^-CD14^-CD19^-CD4^+CD45RA^-CXCR5^{Hi}PD^{Hi}$ cells. Memory B cells (3×10^5 cells per well) were cultured with autologous T_{FH} or GC-T_{FH} cells. Allogeneic in vitro differentiated T_{reg} cells were then added to each respective well. All the cell populations were cultured at a 1:1:1 ratio in the presence of SEB (100 ng/ml; Toxin Technology) and complete medium [R10 with 1% GlutaMax (Gibco), 1 mM sodium pyruvate (Gibco), 1% nonessential amino acids (Gibco), 55 μM β-mercaptoethanol (Gibco), and 10 mM HEPES (Gibco)] at 37°C and 5% CO_2. B cell counts and frequencies of live plasmablast ($CD4^-CD19^+CD20^{-/Lo}CD38^+$ cells) were measured after 7 days by flow cytometry. Immunoglobulin concentrations (IgG and IgA) were determined in the supernatants by enzyme-linked immunosorbent assay.

RNA sequencing

To define T_{fr} signature genes, naive $CXCR5^-$ T_{reg} cells were sorted from four cord blood samples, as described above, and tissue T_{fr} cells were sorted from two fresh pediatric tonsils (10- and 7-year-old females) as live $CD20^-CD4^+CD45RO^+CD69^+ICOS^+CD127^{-/Lo}CD25^{Hi}CXCR5^+$ cells. The purity of the sorted cells was confirmed by FOXP3 staining (>97% $FOXP3^+$ cells for naive T_{reg} and >92% $FOXP3^+$ cells for T_{fr} cells). To analyze the transcriptome of the in vitro differentiated T_{reg} cells, we differentiated naive T_{reg} cells from five cord blood samples with IL-12 as described above. On day 2, cells were split into two wells, and fresh AIM-V medium (without additional cytokines) was added to bring the volume of each well to 200 μl. Twelve hours later, cells were harvested for RNA-seq analysis.

After sorting or in vitro culture, cells were transferred to DNA LoBind tubes (Eppendorf), centrifuged, resuspended in RLT buffer (RNeasy Plus Micro kit, Qiagen), vortexed for 10 s, and immediately stored at −80°C before RNA isolation. Total RNA was extracted with RNeasy plus micro kit (Qiagen) following the manufacturer's instructions and eluted in 14 μl of ribonuclease (RNase)–free water. RNA quality was assessed using a 2200 TapeStation (Agilent Technologies) and a High Sensitivity RNA ScreenTape (Agilent Technologies). All samples showed RNA integrity number (RIN) > 8.5. RNA quantity was evaluated by Qubit RNA HS Assay Kit (Thermo Fisher Scientific). For every sample, 1 to 10 ng of purified total RNA was converted into cDNA and amplified (9 to 12 cycles) using the SMART-Seq HT kit (Takara Bio) or the SMART-Seq v4 Ultra Low Input RNA kit (Takara Bio) for T_{fr} analyzed ex vivo or after the in vitro differentiation, respectively. Ten nanograms of cDNA was used to prepare DNA libraries using Nextera XT DNA Library Preparation Kit (96 samples, Illumina) and Nextera XT Index Kit (96 indexes, Set A, Illumina). Next, barcoded samples were pooled, diluted at 1.8 pM, and loaded onto a NextSeq 500/550 High Output Kit v2.5 flow cell (75 cycles, 120 million to 300 million of reads, Illumina). Single-end sequencing was performed on a NextSeq 550 (Illumina).

CUT&RUN

Freshly isolated naive T_{reg} cells and naive conventional CD4 T cells (7.5×10^4 cells per well) from cord blood were cultured at 37°C in serum-free AIM-V with 2 μl of Dynabeads Human T-Activator CD3/CD28 and IL-2 (5 ng/ml; R&D Systems) for T_{reg} cells or IL-7 (4 ng/ml) for conventional CD4 T cells. After 2.5 days, cells were washed and cultured at 37°C in serum-free AIM-V for 60 min with IL-12 (20 ng/ml; R&D Systems). The cells were harvested, washed with cold PBS, and kept on ice until processing.

CUT&RUN was performed as per Epicypher's Cutana CUT&RUN protocol (https://epicypher.com/content/documents/protocols/cutana-cut&run-protocol-2.1.pdf) with some modifications. A 1X Roche cOmplete EDTA-free protease inhibitor and PhosSTOP phosphatase inhibitor were added to all buffers. Cells were cross-linked in 0.1% formaldehyde for 1 min. The reaction was quenched by adding glycine to a final concentration of 125 mM. Cells were then washed with PBS and counted with a hemocytometer. Nuclei were isolated via a 10-min incubation in cold nuclear extraction buffer [20 mM HEPES (pH 7.9), 10 mM KCl, 0.1% Triton X-100, 20% glycerol, and 0.5 mM spermidine] followed by centrifugation at 1800*g* at room temperature. The supernatant was discarded, and nuclei were resuspended in 100 μl of nuclear extraction buffer per condition. Nuclei were bound to concanavalin A–coated magnetic beads (Bangs laboratories) in bulk, and then an equal volume of the bead-cell slurry was aliquoted into separate tubes per condition. At this point, nuclei were either frozen in an isopropanol-filled chiller at −80°C and later thawed at 37°C or used fresh for downstream steps. After bead binding, the supernatant was discarded, and the bead-cell slurry was resuspended in 250 μl of dig-wash buffer [0.01% digitonin, 20 mM HEPES (pH 7.5), 150 mM NaCl, and 0.5 mM spermidine] with 2 mM EDTA containing primary STAT4 antibody or IgG control (table S2). Antibody incubation was carried out overnight at 4°C on a rotator. Samples were washed with dig-wash buffer

to remove unbound antibodies and then incubated with protein A-micrococcal nuclease (A–MNase, 700 ng/ml; kind gift of the Sarma lab) in dig-wash buffer for 10 min at room temperature on a nutator. Samples were washed with dig wash buffer and resuspended in 50 μl of dig wash buffer and then cooled to 0°C on a metal tube rack on ice for 5 min. One microliter of $CaCl_2$ (100 mM) was added per sample to activate the MNase, and samples were digested for 2 hours at 4°C on a nutator. Thirty-three microliters of stop buffer [340 mM NaCl, 20 mM EDTA, 4 mM EGTA, RNase A (50 μg/ml), and glycogen (50 μg/ml)] was added to each reaction. Samples were incubated for 30 min at 37°C to release cleaved chromatin fragments. The supernatants containing liberated chromatin were transferred to fresh tubes, and DNA was purified via phenol-chloroform isoamyl acetate extraction followed by ethanol precipitation. Purified DNA was quantified via the Qubit dsDNA HS Assay (Thermo Fisher Scientific) and then used to construct libraries via the NEBNext Ultra II DNA Library Prep Kit for Illumina. Paired-end sequencing was performed on a NextSeq 1000 (Illumina).

Statistical analysis

Statistical analysis was conducted in GraphPad Prism version 8. Significance of differences among groups was calculated with nonparametric paired Wilcoxon test, Mann-Whitney test, or parametric two-way analysis of variance (ANOVA) test according to the experimental design and data distribution. The precise number of samples analyzed in each experiment and the respective statistical test used for analysis are reported in figure captions. Statistical significance was set at the critical values of $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), and $P < 0.0001$ (****).

Supplementary Materials

The PDF file includes:
Materials and Methods
Figs. S1 to S7
Tables S1 to S3

Other Supplementary Material for this manuscript includes the following:
Data files S1 to S3
MDAR Reproducibility Checklist

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