

# TSLP-activated dendritic cells induce human T follicular helper cell differentiation through OX40-ligand

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**T follicular helper cells (Tfh) are important regulators of humoral responses. Human Tfh polarization pathways have been thus far associated with Th1 and Th17 polarization pathways. How human Tfh cells differentiate in Th2-skewed environments is unknown. We show that thymic stromal lymphopoietin (TSLP)-activated dendritic cells (DCs) promote human Tfh differentiation from naive CD4 T cells. We identified a novel population, distinct from Th2 cells, expressing IL-21 and TNF, suggestive of inflammatory cells. TSLP-induced T cells expressed CXCR5, CXCL13, ICOS, PD1, BCL6, BTLA, and SAP, among other Tfh markers. Functionally, TSLP-DC-polarized T cells induced IgE secretion by memory B cells, and this depended on IL-4R $\alpha$ . TSLP-activated DCs stimulated circulating memory Tfh cells to produce IL-21 and CXCL13. Mechanistically, TSLP-induced Tfh differentiation depended on OX40-ligand, but not on ICOS-ligand. Our results delineate a pathway of human Tfh differentiation in Th2 environments.**

## INTRODUCTION

Differentiation of naive CD4 T cells into specialized T helper (Th) lymphocyte subsets is crucial to immune responses (O'Shea and Paul, 2010). Among Th subsets, T follicular helper cells (Tfh) have been characterized for their role in B cell help (Tangye et al., 2013). Tfh cells express specific sets of secreted and surface molecules, comprising IL-21, CXCL13, ICOS, PD1, and CXCR5, which provide important signals for B cell survival and maturation in the germinal centers (GCs; Kim et al., 2004; Crotty, 2014).

The Th1-inducing cytokine IL-12 promotes human Tfh polarization (Trinchieri, 2003; Schmitt et al., 2009). Mutations in the *IL-12R $\beta$*  downstream pathway affect IL-21 production and Tfh generation in humans (Ma et al., 2012). IL-27, another Th1-inducing factor, can induce human Tfh polarization (Gringhuis et al., 2014). The cytokine cocktail

used to polarize in vitro human Th17 cells, and in particular TGF- $\beta$ , can promote Tfh development as well (Schmitt et al., 2014). Altogether, these data led to the hypothesis that in humans Tfh polarization is preferentially associated with Th1 and Th17 polarizing environments (Ueno et al., 2015).

Tfh cells have been described in Th2-dominated environments, such as allergy (Kemeny, 2012), and in the absence of Th1 and Th17 polarization (Glatman Zaretsky et al., 2009; Liang et al., 2011; Tangye et al., 2013). However, IL-4, the master Th2 cytokine, inhibits human Tfh differentiation (Schmitt et al., 2014). This raises the important question of how Tfh differentiation can occur in Th2-dominated environments in humans.

We hypothesized that the epithelial-derived cytokine thymic stromal lymphopoietin (TSLP) might play a role in Tfh cell polarization. Independent evidences make TSLP a strong candidate for Tfh polarization. First, TSLP is highly expressed in different Th2-dominated environments, such as airways of asthmatic patients, mucosal tissues in helminth

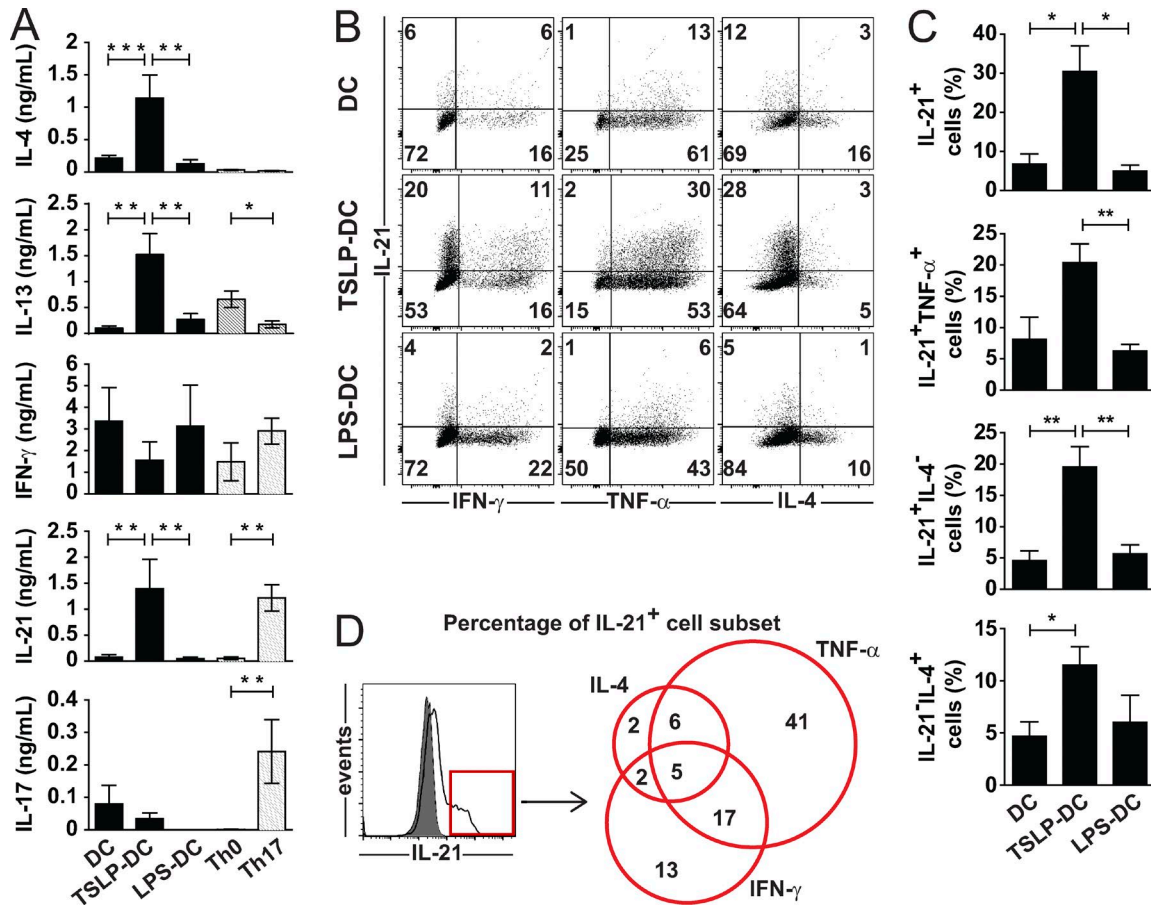
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Abbreviations used: AD, atopic dermatitis; CBA, cytometric bead array; CD40L, CD40 ligand; GC, germinal center; ICOS, Inducible costimulator; MFI, mean fluorescence intensity; NS, Netherton syndrome; OX40L, OX40 ligand; pDC, plasmacytoid DC; Tfh, T follicular helper; Th, T helper; TSLP, thymic stromal lymphopoietin.

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**Figure 1. TSLP-activated DCs polarize naive CD4 T cells into IL-21-secreting cells.** Untreated DCs, treated with TSLP (TSLP-DC) or LPS (LPS-DC) were cultured with naive CD4 T cells for 6 d. (A) CBA (IL-4, IL-13, IFN- $\gamma$ , and IL-17A) and ELISA (IL-21) assays after 24 h of restimulation with anti CD3/CD28 beads. Th0, naive T cells cultured for 6 d with anti-CD3/CD28; Th17, Th0 plus Th17 polarizing cytokines (IL1 $\beta$ , IL-23, TGF- $\beta$ , and IL-6). Data are mean  $\pm$  SEM from nine independent experiments. (B) Intracellular FACS staining for IL-21, IFN- $\gamma$ , TNF, and IL-4 for one representative donor. Gate is on activated DAPI<sup>-</sup> CD4 T cells. (C) Quantification of data as in B. Data are mean  $\pm$  SEM from six independent experiments. (D) Distribution of IL-21<sup>+</sup> cells (red square) polarized by TSLP-DC coproducing IL-4, TNF, and IFN- $\gamma$ . Filled histogram, isotype control; black line, IL-21 staining. Mean of six independent experiments. Single IL-21 producers (16%) are not plotted. \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001, by Wilcoxon or Student's *t* test.

infections, and AD lesional skin (Soumelis et al., 2002; Ying et al., 2005; Ziegler and Artis, 2010). Both AD and allergic patients present deregulated IgE production (Gould et al., 2003). Second, TSLP is expressed in human tonsils, where GC reactions occur (Liu et al., 2007). Third, TSLP contributes to Th2 polarization through DC activation, and induces an inflammatory Th2 response (Soumelis et al., 2002). Fourth, TSLP-activated DCs express OX40 ligand (OX40L), which has been linked to Tfh polarization (Jacquemin et al., 2015).

In this work, we establish a novel Tfh differentiation pathway driven by TSLP. We dissect an axis linking TSLP, DCs, T cells, B cells, and IgE production.

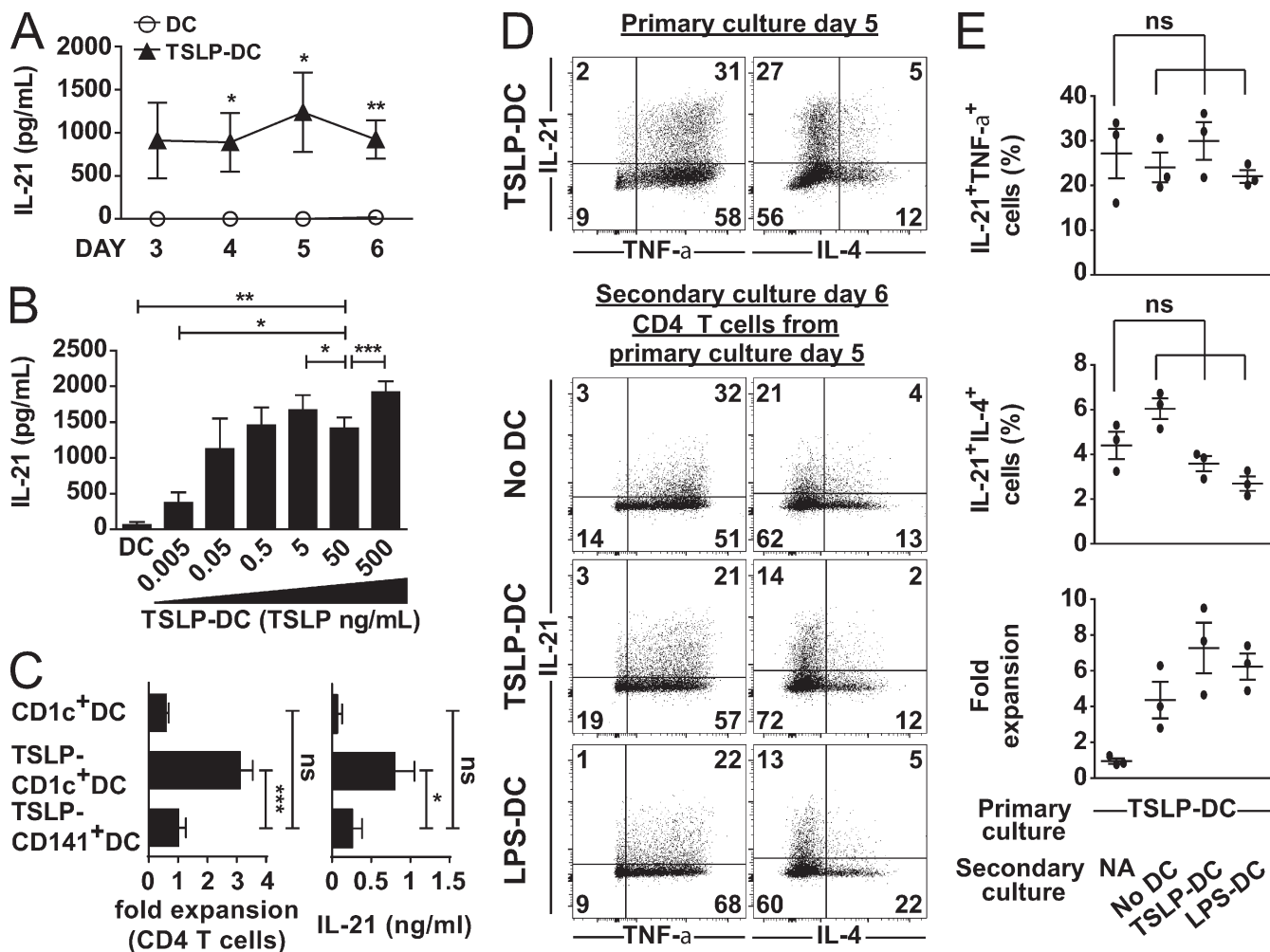
**RESULTS**

**TSLP-activated DCs polarize naive CD4 T cells into IL-21-secreting cells**

We used primary DCs from human blood activated with TSLP (TSLP-DC) to differentiate naive CD4 cells into

Th cells in an allogeneic system. As expected, after 6 d of co-culture, TSLP-DC induced Th cells that secreted IL-4 and IL-13, but low levels of IFN- $\gamma$ , which are features of Th2 polarization (Fig. 1 A; Soumelis et al., 2002; Ziegler and Artis, 2010). To separate the effect of TSLP-induced activation from an intrinsic property of human blood DCs, we used nonactivated DCs as a negative control. As an additional control, we used LPS-activated DCs (LPS-DC), which induced IFN- $\gamma$  but low IL-4 and IL-13 secretion from T cells (Fig. 1 A), in accordance with Th1 polarization.

Surprisingly, TSLP-DC polarized naive CD4 T cells to produce high amounts of IL-21 (Fig. 1 A). The amount of IL-21 induced by TSLP-DC polarization was similar to that of in vitro polarized Th17 cells. We recently showed that TSLP synergizes with CD40L in DCs to promote the expression of the Th17-polarizing cytokine IL-23 (Volpe et al., 2014). TSLP-DC induced low and inconsistent IL-17A secretion by CD4 T cells in comparison with in vitro-polarized Th17,

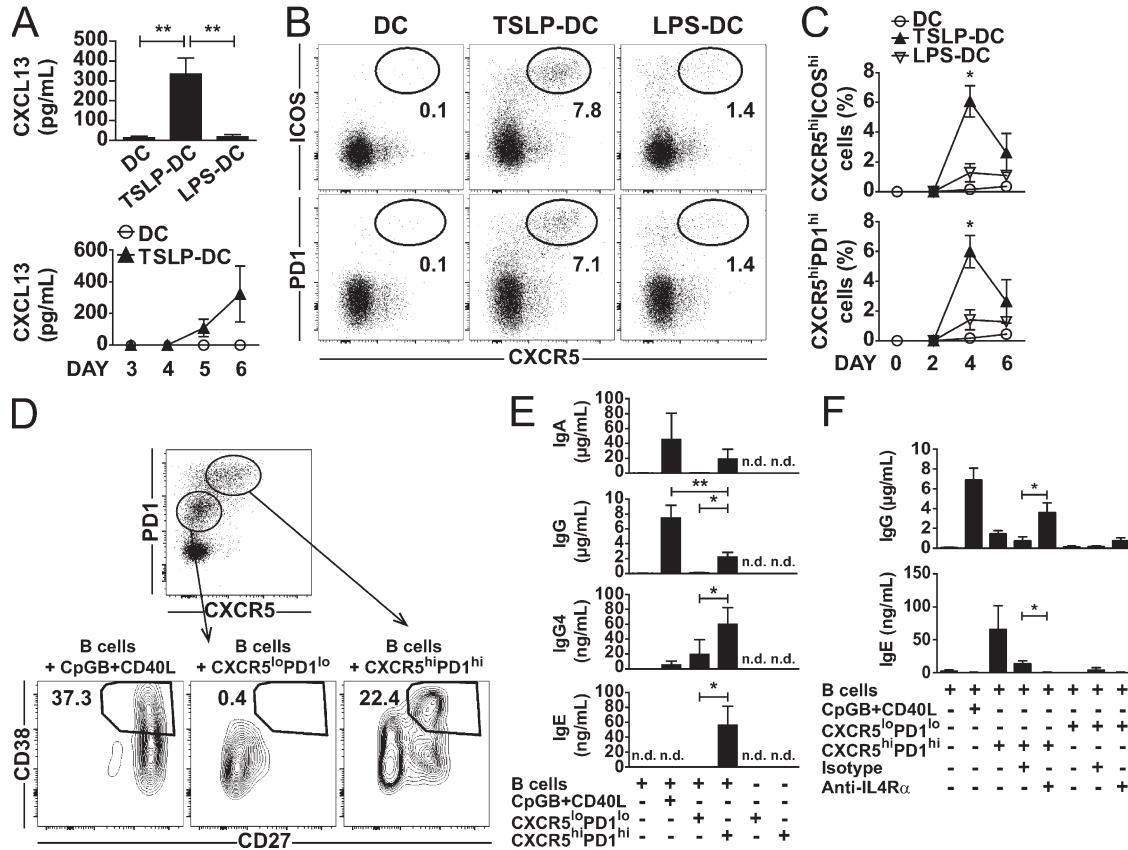


**Figure 2. IL-21 production by TSLP-DC-polarized CD4 T cells is stable.** (A) DCs were activated with TSLP (50 ng/ml, TSLP-DC, filled triangles) or in control medium (DC, circles). After 24 h, DCs were co-cultured with naive CD4 T cells and stimulated for 24 h with anti-CD3/CD28 beads. IL-21 concentration in the supernatants from seven independent experiments. (B) Quantification of IL-21 secretion by CD4 T cells polarized for 6 d with DCs, previously activated for 24 h with increasing doses of TSLP. SEM for four independent experiments; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , paired Student's  $t$  test. (C) CD4 T cell fold expansion and IL-21 secretion from co-cultures with untreated CD1c<sup>+</sup>, TSLP-activated CD1c<sup>+</sup> and TSLP-activated CD141<sup>+</sup> DCs. SEM for 12 independent experiments; \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ , paired Student's  $t$  test. (D) Intracellular FACS staining of IL-21, TNF, and IL-4 by TSLP-DC-activated CD4 T cells at the indicated days of primary and secondary culture from a representative CD4 T cell donor. In primary culture CD4 T cells were activated by TSLP-DC. In secondary culture, cells from day 5 of primary culture were cultured for 6 d in medium alone (No DC), with TSLP-DC or LPS-DC. (E) Percentage of IL-21<sup>+</sup>TNF<sup>+</sup> and IL-21<sup>+</sup>IL-4<sup>+</sup> cells (among activated cells) and fold expansion in primary and secondary culture as indicated, in three independent experiments. NA, not applicable.

excluding a strong Th17 polarization by TSLP-DC. To check whether TSLP could act directly on CD4 T cell, in addition to DCs, we analyzed by FACS the expression of TSLP receptor (R) chains (TSLPR and IL-7R $\beta$ ) in naive CD4 T cells and DCs. DCs expressed high levels of both chains, whereas ex vivo or activated (5 d of anti CD3/CD28 beads, Th0 cells) naive CD4 T cells expressed IL7R $\alpha$  but inconsistent levels of TSLPR (Fig. S1 A). We cultured sorted naive CD4 T cells with anti-CD3/CD28 beads and TSLP, in the absence of DCs. After 6 d of culture, we did not detect any induction of IL-21 by Th0 cells cultured either with or without TSLP. As

a control, we detected IL-21 production by in vitro polarized Th17 (Fig. S1 B). Therefore, we concluded that TSLP was inducing IL-21 production by CD4 T cells through DCs.

Next, we investigated whether IL-21 was coproduced with other cytokines at the single T cell level. We performed intracellular staining for IL-21, in combination with IFN- $\gamma$ , IL-4, and TNF as features of inflammatory Th2 differentiation induced by TSLP (Ito et al., 2005). At day 6 of co-culture, ~30% of the CD4 T cells activated by TSLP-DC were positive for IL-21, indicating a strong IL-21 polarization. IL-21 was mainly co-produced with TNF (20% of activated CD4



**Figure 3. T cells polarized by TSLP-DC possess key features of human Tfh cells.** (A) ELISA for CXCL13 production by CD4 T cells differentiated for 6 d in co-culture with DCs, TSLP-DC, or LPS-DC. Cytokines secretion was measured after an additional 24 h of anti-CD3/CD28 bead stimulation. Data are mean ± SEM from 20 independent experiments. \*\*, P < 0.01, paired Student's *t* test. For the kinetic of CXCL13 expression, CD4 T cells were restimulated for 24 h with anti-CD3/CD28 beads after 3, 4, 5, or 6 d of co-culture with DCs (circles) or TSLP-DC (triangles). SEM for seven independent experiments. (B) FACS staining for ICOS, PD1, and CXCR5 in CD4 T cells after 4 d of co-culture with DCs. CXCR5<sup>hi</sup>/ICOS<sup>hi</sup> and CXCR5<sup>hi</sup>/PD1<sup>hi</sup> cells within CD4 T DAPI<sup>-</sup> cells from a representative donor are shown. (C) Quantification of cell populations as indicated in B in naive CD4 T cells after 0, 2, 4, or 6 d of co-culture with DCs (circles), TSLP-DC (filled triangles), or LPS-DC (open triangles). SEM from six independent experiments. (D) CXCR5<sup>hi</sup>/PD1<sup>hi</sup> and CXCR5<sup>lo</sup>/PD1<sup>lo</sup> CD4 T cells polarized 4 d by TSLP-DC were sorted (top), and co-cultured with autologous memory B cells for 14 d. CD38 and CD27 were measured by FACS on B cells (DAPI<sup>-</sup>/CD3<sup>-</sup>/CD4<sup>-</sup>/CD19<sup>+</sup>). One representative plot is shown. (E) IgA, IgG, IgG4, and IgE were quantified in the supernatants of co-cultures, as in D, in the indicated conditions. Mean ± SEM for five donors. n.d., not detected. (F) Quantification of IgG and IgE in the supernatants of memory B cells co-cultured as in D, plus IL4R-α blocking or isotype control antibodies. SEM from five independent experiments are plotted. \*, P < 0.05; \*\*, P < 0.01, paired Student's *t* test.

cells). We identified IL-21<sup>+</sup>IL-4<sup>-</sup> (20%) and IL-21<sup>-</sup>IL-4<sup>+</sup> (12%) populations, suggesting that distinct Th subsets arise in the presence of TSLP-DC (Fig. 1, B and C). To better characterize the cytokine expression pattern of IL-21<sup>+</sup> CD4 T cells after 6 d of co-culture with TSLP-DC, we calculated the percentage of cells coexpressing different combinations of cytokines. Among the IL-21<sup>+</sup> cells, we detected a small population (5%) of Th cells coexpressing TNF, IL-4, and IFN-γ (Fig. 1 D). The majority (69%) of Th cells expressed IL-21 in combination with TNF (Fig. 1 D).

Next, we examined IL-21 induction by TSLP-DC in CD4 T cells. We detected IL-21 secretion after 3 d of DC/T cell co-culture (Fig. 2 A), using as low as 5 pg/ml TSLP to activate DCs (Fig. 2 B). We separately activated the CD1c<sup>+</sup> and CD141<sup>+</sup> DC subsets with TSLP. After co-culture with

naive CD4 T cells, we observed that TSLP CD1c<sup>+</sup> DCs induced higher CD4 T cell expansion and IL-21 production, as compared with CD141<sup>+</sup> DCs. We did not measure any significant difference between nontreated CD1c<sup>+</sup> DCs and TSLP-CD141<sup>+</sup> DCs with regards to IL-21 production and CD4 T cell expansion (Fig. 2 C).

Next, we investigated the stability of IL-21, TNF, and IL-4 expression by TSLP-DC-activated CD4 T cells. We compared the intracellular expression of these cytokines by CD4 T cells cultured for 5 d in the presence of TSLP-DC (Fig. 2 D, primary culture), with the same CD4 T cells recultured for additional 6 d in medium without DCs (No DC), with TSLP-DC or LPS-DC (Fig. 2 D, secondary culture). The percentages of IL-21<sup>+</sup>TNF<sup>+</sup> and IL-21<sup>+</sup>IL-4<sup>+</sup> cells were comparable between the primary and secondary culture (Fig. 2 E), suggesting that the

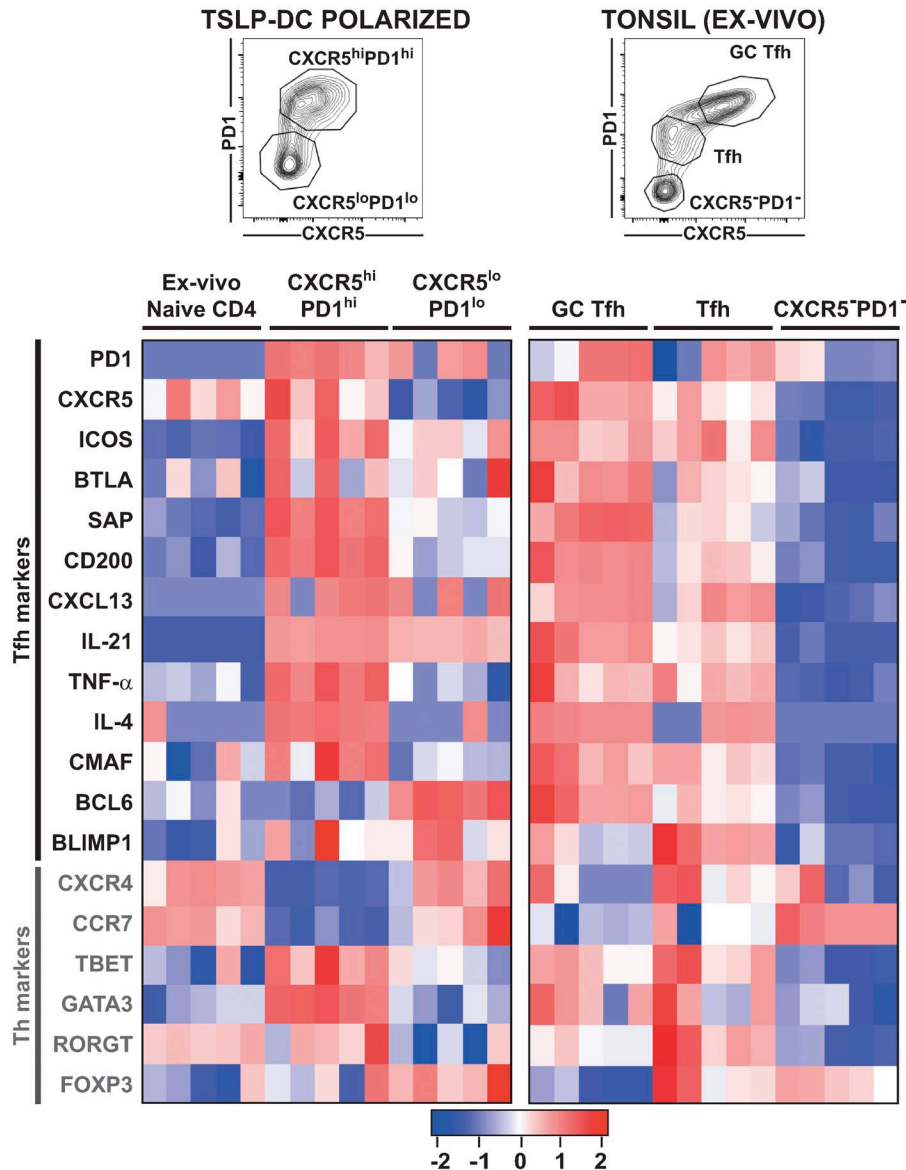


Figure 4. T cells polarized by TSLP-DC show an expression pattern similar to tonsillar Tfh cells. Heat map showing mRNA quantification of Tfh and Th markers in naive CD4 T cells, TSLP-DC-polarized CD4 T cells, and human tonsillar CD4 populations. CD4 T cells differentiated for 4 d with TSLP-DC were sorted as indicated (top left). Three populations of tonsillar CD4 cells were sorted (top right): CXCR5<sup>hi</sup>/PD1<sup>hi</sup> (GC Tfh), CXCR5<sup>int</sup>/PD1<sup>int</sup> (Tfh), and CXCR5<sup>-</sup>/PD1<sup>-</sup>. mRNA levels normalized on the B2M and RPL34 housekeeping genes and center reduced are displayed on the heat map from five independent donors and two independent experiments.

expression of these cytokines was stable. Additionally, by counting live cells we found that CD4 T cells activated by TSLP-DC expanded, even in the absence of further DC stimulation (Fig. 2 E, bottom). These two results suggested an overall expansion of the IL-21<sup>+</sup>TNF<sup>+</sup> and IL-21<sup>+</sup>IL-4<sup>+</sup> cell populations.

Thus, TSLP-DC promoted the generation of a stable, novel Th subset producing IL-21 and TNF, in combination or not with the Th2 cytokine IL-4.

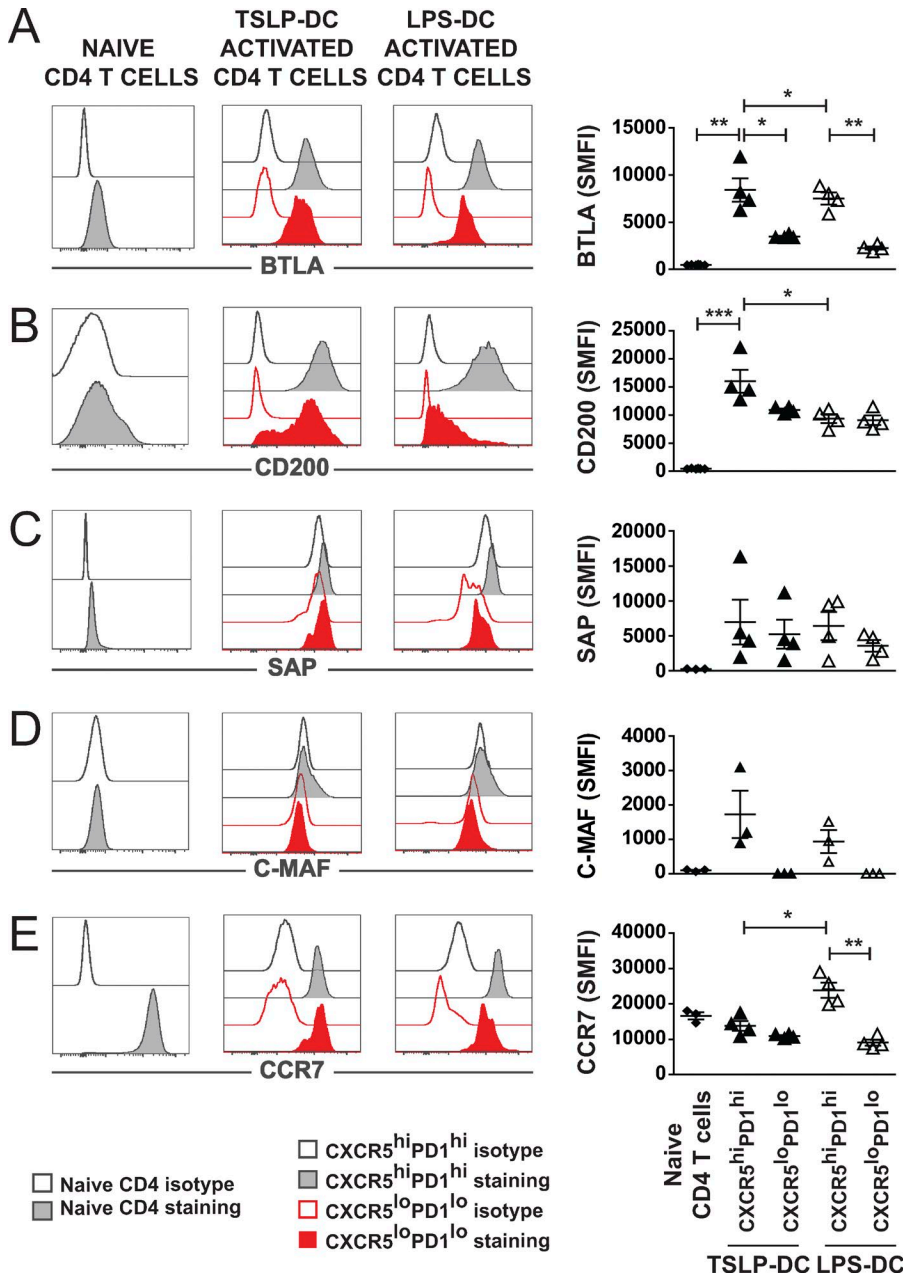
#### Th cells polarized by TSLP-DC possess features of human Tfh cells

Because IL-21 is highly produced by Tfh cells (Schmitt et al., 2014), we asked whether TSLP-DC-polarized T helper cells had Tfh markers.

We measured the secretion of CXCL13, a chemokine produced by Tfh but not by other Th cell subsets (Kim et al.,

2004), after 6 d of co-culture followed by 24 h of anti-CD3/CD28 stimulation. TSLP-DC, but not unstimulated DCs or LPS-DC, induced the secretion of CXCL13 by CD4 T cells (Fig. 3 A, top), suggesting Tfh polarization. CXCL13 secretion was detectable from day 5 of co-culture (Fig. 3 A, bottom).

A feature of human Tfh cells is the expression of high levels of the CXCL13 receptor CXCR5, in combination with high levels of ICOS and PD1 (Bryant et al., 2007; Crotty, 2014). We identified by FACS CXCR5<sup>high</sup>ICOS<sup>hi</sup> and CXCR5<sup>hi</sup>PD1<sup>hi</sup> CD4 T cells after 4 d of co-culture with TSLP-DC (Fig. 3 B). TSLP-DC increased the percentage of CXCR5<sup>hi</sup>ICOS<sup>hi</sup> and CXCR5<sup>hi</sup>PD1<sup>hi</sup> populations at day 4 as compared with day 2 and day 6 of co-culture, and in comparison to nonactivated DC and LPS-DC (Fig. 3 C). The use of naive T cells (CD4<sup>+</sup>CD25<sup>-</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup>) sorted to 99% purity, without detectable CXCR5<sup>+</sup> cells (Fig. 3 C),

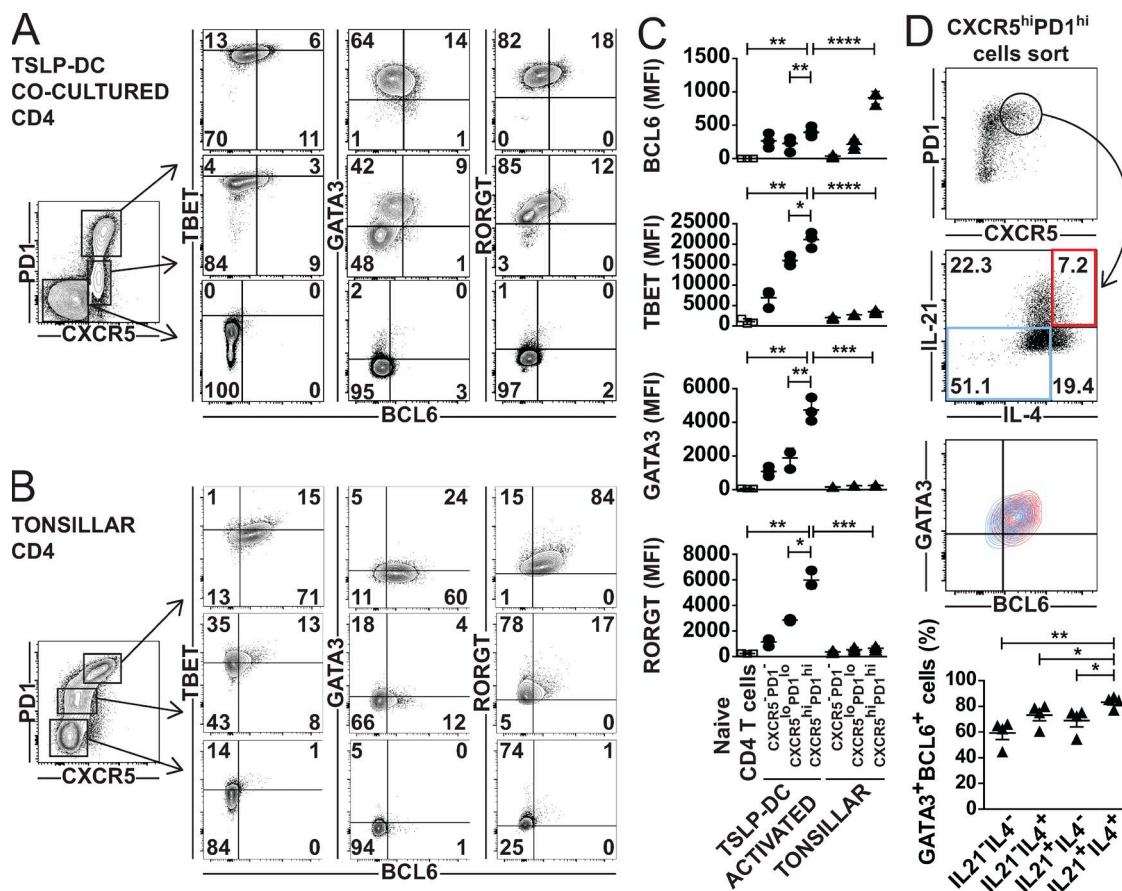


excluded that these cells originated from the rare blood memory Tfh population, characterized by CXCR5 expression (Morita et al., 2011).

One key function of Tfh cells is their ability to help B cells to secrete class-switched Igs (Crotty, 2014). To test whether the CXCR5<sup>hi</sup>PD1<sup>hi</sup> cells induced by TSLP-DC were able to help B cells, we sorted CXCR5<sup>hi</sup>PD1<sup>hi</sup> and CXCR5<sup>lo</sup>PD1<sup>lo</sup> cells after 4 d of co-culture with TSLP-DC, and co-cultured them with autologous memory B cells (Fig. 3 D). We detected CD19<sup>+</sup>CD38<sup>hi</sup>CD27<sup>+</sup> B cells after 14 d of co-culture with CXCR5<sup>hi</sup>PD1<sup>hi</sup>, but not with CXCR5<sup>lo</sup>PD1<sup>lo</sup> T cells polarized by TSLP-DC, similarly to the positive control of memory B cells activated by CD40-Ligand (CD40L) and CpG oligode-

oxynucleotides type B (CpG-B; Fig. 3 D). At the same time point, we measured secretion of class switched Igs in the supernatants. Memory B cells activated with CD40L and CpG-B secreted IgA and IgG, as expected (Bernasconi et al., 2002). TSLP-DC polarized CXCR5<sup>hi</sup>PD1<sup>hi</sup> cells specifically induced IgG4 and IgE secretion by memory B cells. In comparison, IgA, IgG, and IgE secretion in the presence of CXCR5<sup>lo</sup>PD1<sup>lo</sup> cells was low and inconsistent (Fig. 3 E). We measured lower amounts of IgA and IgG, induced by CXCR5<sup>hi</sup>/PD1<sup>hi</sup> cells as compared with memory B cells activated with CD40L and CpG-B (Fig. 3 E), in accordance with selective induction of IgE and IgG4.

Next, we investigated the mechanism by which TSLP-DC-induced CXCR5<sup>hi</sup>PD1<sup>hi</sup> cells promoted IgE se-



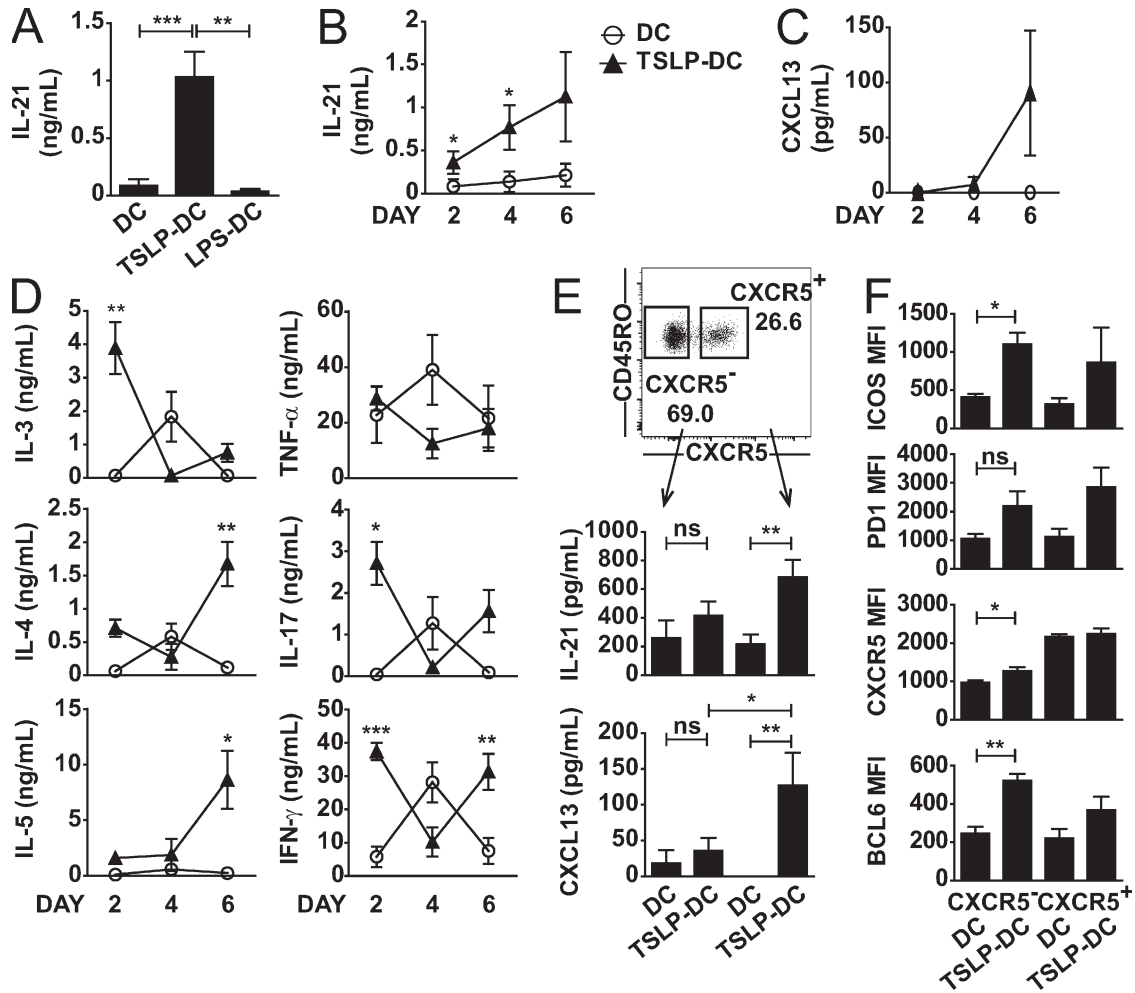
**Figure 6. CD4 T cells activated by TSLP-DC coexpress BCL6 and GATA3.** (A) FACS staining for BCL6, TBET, GATA3, and RORGT in naive CD4 T cells co-cultured with TSLP-DC for 4 d. (B) Tonsillar CD4 cells analyzed as in A. Gates were set using fluorescence minus one plus isotype, and percentage of cells in each quadrant are shown for one representative donor. (C) Quantification of BCL6, TBET, GATA3, and RORGT MFI in naive CD4 T cells and from data shown in A and B from three or five independent experiments. Empty squares, naive CD4 T cells; dots, TSLP-DC activated CD4; triangles, tonsillar CD4<sup>+</sup> T cells. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ , unpaired Student's *t* test. (D) CD4 T cells sorted as CXCR5<sup>hi</sup>PD1<sup>hi</sup> at day 4 of co-culture with TSLP-DC were analyzed for intracellular expression of IL-4, IL-21, GATA3, and BCL6. One representative experiment is shown, and quantification of % of GATA3<sup>+</sup>/BCL6<sup>+</sup> cells is plotted for four independent experiments. Mean  $\pm$  SEM is plotted. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , paired Student's *t* test.

cretion by memory B cells. IL-4 mediates IgE production by human B cells (Pène et al., 1988). TSLP-DC-polarized T cells secreted IL-4 (Fig. 1 A). We functionally blocked IL-4 receptor  $\alpha$  (IL-4R $\beta$ ) in the co-culture of TSLP-DC-polarized T cells and memory B cells. After targeting of IL-4R $\beta$  by using a functional blocking antibody, we were unable to detect IgE secretion by memory B cells in the presence of TSLP-DC-polarized CXCR5<sup>hi</sup>PD1<sup>hi</sup> cells. In parallel, we detected an increase in IgG production (Fig. 3 F). As a control, we checked that IL-4R $\beta$  blocking antibody did not decrease B cell viability. Our data are in accordance with previous data showing that IL-4-reduced IgG production by human B cells (Nies et al., 2002).

Altogether, these results show that TSLP-DC induced the polarization of cells expressing Tfh markers such as CXCR5, PD1, and ICOS, and that these cells shared functional features of human Tfh2, comprising the ability to stimulate IgE secretion by B cells. Mechanistically, we showed that IgE induction by TSLP-DC-polarized Tfh cells depended on IL-4R $\beta$ .

### T cells polarized by TSLP-DC show molecular markers similar to tonsillar Tfh

To confirm that TSLP-DC-polarized T cells presented features of Tfh cells, we selected a set of Tfh markers on the basis of transcriptomic analysis of human Tfh cells (Kim et al., 2004). We quantified the expression of these Tfh markers by qPCR on sorted CXCR5<sup>hi</sup>PD1<sup>hi</sup> and CXCR5<sup>lo</sup>PD1<sup>lo</sup> CD4 T cell populations identified among activated T cells after 4 d of co-culture with TSLP-DC (Fig. 4, top left). As a comparison, we analyzed sorted naive CD4 T cells. CXCR5<sup>hi</sup>PD1<sup>hi</sup> cells expressed higher levels of Tfh markers at the mRNA level (BTLA, CXCR5, CXCL13, ICOS, PD1, SAP, CD200, and C-MAF) as compared with CXCR5<sup>lo</sup>PD1<sup>lo</sup> cells (Fig. 4 and Fig. S2). Additionally, CXCR5<sup>hi</sup>/PD1<sup>hi</sup> cells polarized by TSLP-DC expressed higher mRNA levels of the cytokines IL-21, IL-4, and TNF, as compared with CXCR5<sup>lo</sup>/PD1<sup>lo</sup> cells (Fig. 4). As expected, naive CD4 T cells did not express significant levels of Tfh markers.



**Figure 7. Memory CD4 T cells express Tfh factors after activation by TSLP-DC.** CD4 memory T cells were cultured with DCs, TSLP-DC, or LPS-DC, and cytokines were measured at the indicated days after 24 h of restimulation with anti-CD3/CD28 beads. ELISA assay for IL-21 at day 6 of culture in A and at day 2, 4, and 6 in B. Mean  $\pm$  SEM for 13 and 5 donors is shown, from four and two independent experiments, respectively. ELISA assay for CXCL13 in C and CBA assay for Th cytokines in D in the same experimental settings as in B. (E and F) Memory CD4 T cells were separated into CXCR5<sup>+</sup> and CXCR5<sup>-</sup> cells by FACS sorting, and cultured with DCs or TSLP-DC for 6 d. IL-21 and CXCL13 quantification after 24 h of anti-CD3/CD28 stimulation is shown as mean  $\pm$  SEM from nine independent experiments. FACS staining for ICOS, PD1, and CXCR5 at day 6 of co-culture. BCL6 was quantified on the CXCR5<sup>hi</sup>/PD1<sup>hi</sup> population. The geometric MFI is plotted for three or two independent experiments. \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001, paired Student's *t* test.

Notably, the expression pattern of Tfh markers paralleled the one of our positive controls, represented by sorted tonsillar GC Tfh (CD4<sup>+</sup>CD45RO<sup>+</sup>CXCR5<sup>hi</sup>PD1<sup>hi</sup>) and Tfh (CD4<sup>+</sup>CD45RO<sup>+</sup>CXCR5<sup>lo</sup>PD1<sup>lo</sup>), and differed from non-Tfh CD4 (CD4<sup>+</sup>CD45RO<sup>+</sup>CXCR5<sup>-</sup>PD1<sup>-</sup>; Fig. 4). TSLP-induced CXCR5<sup>hi</sup>/PD1<sup>hi</sup> cells down-regulated the lymph node homing receptors CXCR4 and CCR7 at the mRNA level, suggesting peripheral effector functions. As a control naive CD4 T cells expressed CCR7 and CXCR4 mRNA.

Additionally, we measured the expression of BCL6, BLI MP, TBET, GATA3, RORGT, and FOXP3, transcription factors orchestrating Th subset differentiation. The mRNA expression of BCL6, a transcriptional repressor important for Tfh polarization, was lower in CXCR5<sup>hi</sup>PD1<sup>hi</sup> than

in CXCR5<sup>lo</sup>PD1<sup>lo</sup> cells polarized by TSLP-DC (Fig. 4). CXCR5<sup>hi</sup>PD1<sup>hi</sup> cells induced by TSLP-DC expressed higher levels of TBET, GATA3, and RORGT when compared with CXCR5<sup>lo</sup>PD1<sup>lo</sup> cells, similarly to tonsillar GC Tfh cells. Additionally, the CXCR5<sup>hi</sup>PD1<sup>hi</sup> cells induced by TSLP-DC did not express FOXP3, indicating that they were distinct from regulatory Tfh.

We measured by FACS analysis the expression of BTLA, CD200, SAP, and C-MAF proteins in naive CD4 T cells, as well as in CXCR5<sup>hi</sup>PD1<sup>hi</sup> and CXCR5<sup>lo</sup>PD1<sup>lo</sup> cells, after 4 d of co-culture in the presence of TSLP-DC and LPS-DC as a comparison (Fig. 5, A–D). CXCR5<sup>hi</sup>PD1<sup>hi</sup> cells induced by TSLP-DC expressed BTLA, CD200, SAP, and C-MAF at the protein level, thus validating our mRNA analysis. At the



protein level, we did not measure any significant difference in CCR7 protein levels between TSLP-induced CXCR5<sup>hi</sup>PD1<sup>hi</sup> and CXCR5<sup>lo</sup>PD1<sup>lo</sup>. However, CCR7 protein expression was significantly higher in LPS-DC-induced CXCR5<sup>hi</sup>PD1<sup>hi</sup> cells as compared with TSLP-DC-induced (Fig. 5 E). As a negative control, naive CD4 T cells did not express BTLA, CD200, SAP, or C-MAF protein. As expected, naive CD4 T cells expressed surface CCR7 at similar levels that TSLP-induced CXCR5<sup>hi</sup>PD1<sup>hi</sup> and CXCR5<sup>lo</sup>PD1<sup>lo</sup> cells.

Overall, CXCR5<sup>hi</sup>PD1<sup>hi</sup> cells polarized by TSLP-DC express markers characteristic of human tonsillar Tfh, suggesting that TSLP-DC are able to induce Tfh polarization from naive CD4 T cells.

### CXCR5<sup>hi</sup>PD1<sup>hi</sup> cells polarized by TSLP-DC coexpressed BCL6 and GATA3

Our data showing the expression of BCL6, TBET, GATA3, and RORGT by TSLP-DC activated CD4 T cells are relevant to the coexistence of Th and Tfh polarization programs in a single cell. Therefore, we investigated their coexpression at the single-cell level.

We measured by intracellular FACS staining the expression of BCL6, TBET, GATA3, and RORGT in CD4 T cells co-cultured for 4 d with TSLP-DC. We included, as a negative control, a CXCR5<sup>-</sup>PD1<sup>-</sup> population corresponding to cells that were co-cultured with TSLP-DC but did not display an activated profile (Fig. 6 A, bottom). We compared the expression of the same transcription factors in human tonsillar CD4 populations, identified by different expression levels of CXCR5 and PD1 (Fig. 6 B). TSLP-induced CXCR5<sup>hi</sup>PD1<sup>hi</sup> cells expressed significantly higher levels of BCL6, TBET, GATA3, and RORGT protein when compared with CXCR5<sup>lo</sup>PD1<sup>lo</sup> cells and naive CD4 T cells (Fig. 6, A and C). CXCR5<sup>hi</sup>PD1<sup>hi</sup> tonsillar cells expressed higher levels of BCL6, but lower levels of TBET, GATA3, and RORGT, as compared with TSLP-induced CXCR5<sup>hi</sup>PD1<sup>hi</sup> cells. These data validated that TSLP-DC-activated T cells expressed higher levels of lineage defining transcription factors, as suggested by our mRNA analysis of Fig. 4. CXCR5<sup>hi</sup>PD1<sup>hi</sup> cells, induced by TSLP-DC, expressed higher levels of BCL6 protein (Fig. 6 A) but lower levels of BCL6 mRNA (Fig. 4) as compared with CXCR5<sup>lo</sup>/PD1<sup>lo</sup> cells. One interpretation of this discrepancy is that there are some differences at the posttranscriptional level between CXCR5<sup>hi</sup>/PD1<sup>hi</sup> and CXCR5<sup>lo</sup>/PD1<sup>lo</sup> cells. Discrepancies between BCL6 mRNA and protein levels have been already reported (Kroenke et al., 2012).

Next, we investigated whether the IL-4<sup>+</sup>IL-21<sup>+</sup> cells we characterized (Fig. 1) coexpressed BCL6 and GATA3. We FACS sorted CXCR5<sup>hi</sup>PD1<sup>hi</sup> cells and analyzed the expression of IL-4, IL-21, BCL6, and GATA3 by intracellular FACS staining. The majority (80%) of IL-4<sup>+</sup>IL-21<sup>+</sup> cells coexpressed BCL6 and GATA3 proteins, as shown by a representative donor and quantification in Fig. 6 D. BCL6/GATA3 double-positive population was significantly enriched in IL-4<sup>+</sup>IL-21<sup>+</sup> cells compared with single cytokine producers

or double-negative cells. This showed that TSLP-DC-activated CXCR5<sup>hi</sup>PD1<sup>hi</sup>IL-21<sup>+</sup>IL-4<sup>+</sup> cells preferentially coexpressed BCL6 and GATA3.

Overall, these data showed that TSLP-DC-induced the expression of BCL6 in combination with Th lineage defining transcription factors, in particular GATA3, at the protein level.

### IL-21 and CXCL13 secretion from memory CD4 T cells are increased by TSLP-DC

TSLP-DC are potent inducers of memory Th2 responses (Wang et al., 2006). To establish whether TSLP-DC stimulated IL-21 and CXCL13 secretion by memory CD4 T cells, we cultured TSLP-DC with allogeneic memory CD4 purified by sorting (99% purity) from healthy donor peripheral blood (CD4<sup>+</sup>CD25<sup>-</sup>CD45RA<sup>-</sup>CD45RO<sup>+</sup>). Memory CD4 T cells secreted increased amounts of IL-21 after 6 d of co-culture in the presence of TSLP-DC, when compared with CD4 memory co-cultured with untreated DCs or LPS-activated DCs (Fig. 7 A).

To gain insight into the dynamic of cytokine secretion by memory T cells activated by TSLP-DC, we washed and restimulated cells after 2, 4 or 6 d of co-culture. IL-21 secretion by memory CD4 T cells was detected after 2 d of co-culture with TSLP-DC, and was higher after 4 and 6 d (Fig. 7 B). CXCL13 was induced at day 6 of co-culture, but barely detectable before (Fig. 7 C). We compared the expression of IL-21 and CXCL13 with the expression of Th2 cytokines (IL-3, IL-4, and IL-5) in the same experiment. IL-3 was induced at day 2, whereas the secretion profiles of IL-4 and IL-5 over time were comparable to the one of CXCL13 (Fig. 7 D, left column). In the same settings, we were unable to detect any statistically significant difference in TNF secretion (Fig. 7 D, right column). The secretion profiles of IL-21 and CXCL13 were different from the ones of IL-17A and IFN- $\gamma$  (Fig. 7 D, right column), characteristics of Th17 and Th1 cells, respectively. This indicated that TSLP-DC-activated memory CD4 T cells to express Tfh cytokines IL-21 and CXCL13 with a kinetic of secretion similar to Th2 cytokines.

Detection of CXCL13 and IL-21 in the co-culture of memory CD4 T cells with TSLP-DC suggested that memory Tfh, which have been described as CXCR5<sup>+</sup>CD4<sup>+</sup>CD45RA<sup>-</sup>CD45RO<sup>+</sup> cells in human peripheral blood (Morita et al., 2011), might be activated by TSLP-DC. To test this hypothesis, we sorted blood memory CD4 based on CXCR5 expression, and co-cultured the CXCR5<sup>-</sup> and CXCR5<sup>+</sup> memory CD4 populations separately with either nonactivated DCs or TSLP-DC (Fig. 7 E, top). TSLP-DC significantly induced IL-21 and CXCL13 secretion by CXCR5<sup>+</sup> memory Tfh in comparison to nonactivated DCs after 6 d of co-culture, followed by 1 d of restimulation with anti-CD3/CD28 beads (Fig. 7 E). At day 6, we measured by FACS the expression of ICOS, PD1, CXCR5, and BCL6 in the same experimental conditions as in Fig. 7 E. TSLP-DC significantly induced ICOS, CXCR5, and BCL6 compared with unstimulated DCs (Fig. 7 F) in memory CXCR5<sup>-</sup> cells.

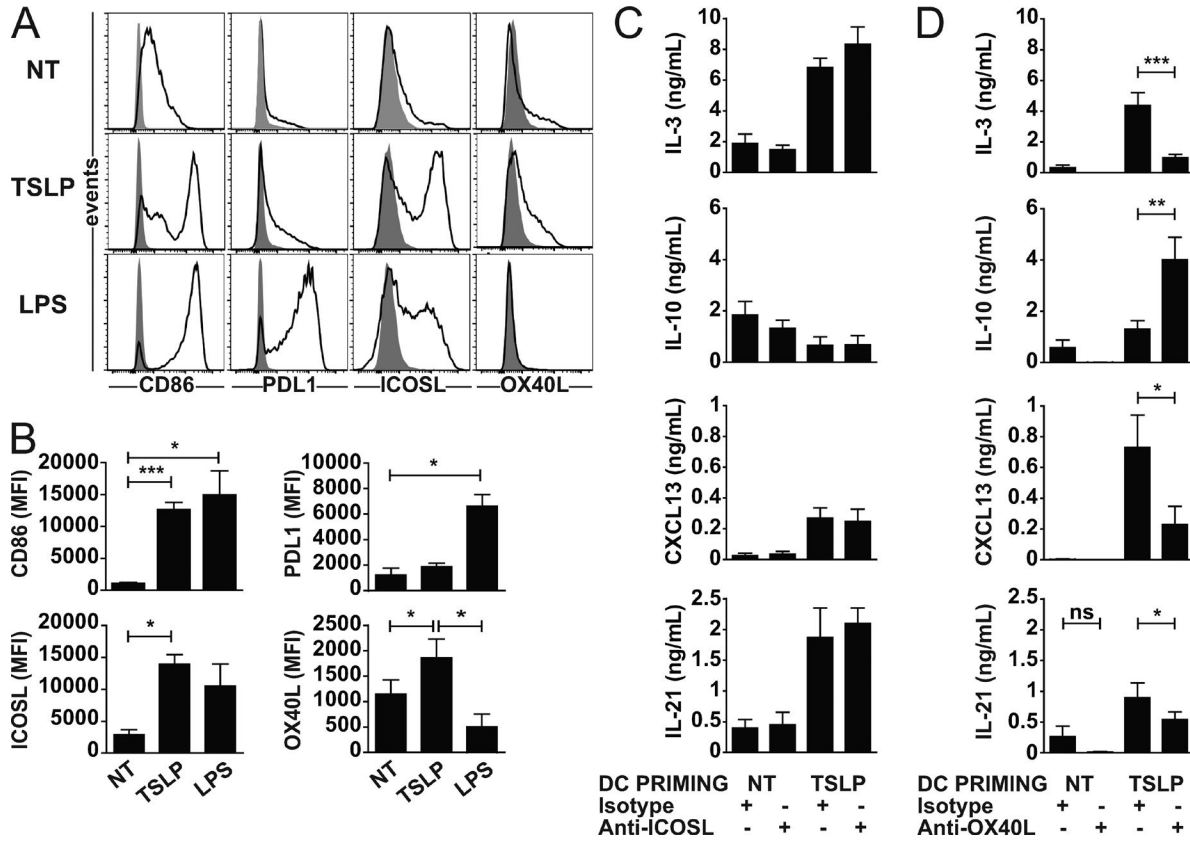


Figure 8. TSLP-DC induce IL-21 and CXCL13 production through OX40L. (A) FACS analysis of surface expression of CD86, PDL1, ICOSL, and OX40L by DCs cultured without any stimulation (NT), TSLP, or LPS for 48 h. Filled gray histogram shows matched isotype control. Black histogram shows antibody staining. One representative donor is shown. (B) Quantification of MFI as in A. Mean  $\pm$  SEM for seven experiments. (C) Quantification of cytokine by CBA (IL-3 and IL-10) or ELISA (CXCL13 and IL-21) by CD4 T cells differentiated during 6 d with DCs or TSLP-DC. Anti-ICOSL blocking antibody or isotype control antibody (25  $\mu$ g/ml) were kept all along the culture. Mean  $\pm$  SEM for four experiments, is plotted. (D) Cells were cultured as in C, and instead of ICOSL blocking antibody, an anti-OX40L antibody or isotype control (50  $\mu$ g/ml) were used. Mean  $\pm$  SEM for seven experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , paired Student's *t* test.

Overall, we concluded that TSLP-DC preferentially stimulated CXCR5<sup>+</sup> CD4 T cells to secrete IL-21 and CXCL13. Additionally, TSLP-DC induced expression of ICOS, CXCR5, and BCL6 on CXCR5<sup>-</sup> memory CD4 T cells.

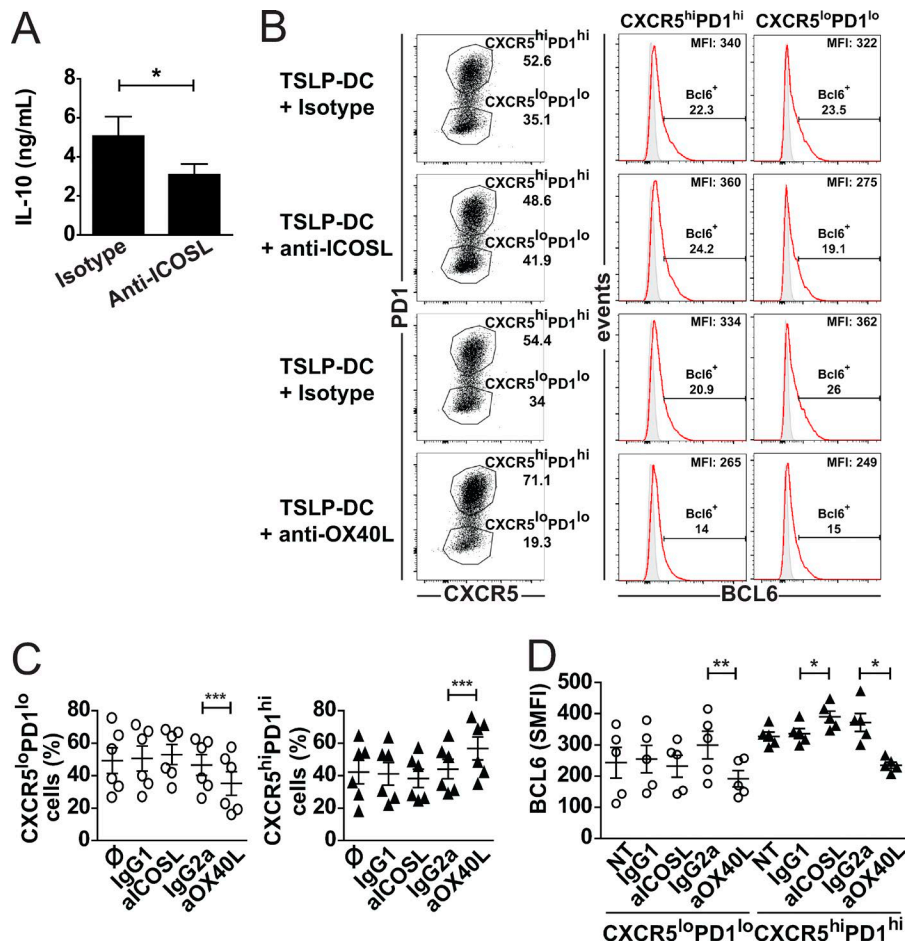
**TSLP-DC induce IL-21 and CXCL13 production through OX40L**

To gain mechanistic insight into TSLP-DC induction of IL-21 and CXCL13 expression, we focused on the Th-polarizing molecules induced by TSLP in DCs. Because TSLP-DC produce low levels of inflammatory cytokines, and no IL-12 (Soumelis et al., 2002), we explored the contribution of surface co-stimulatory molecules associated with Tfh differentiation. We measured by flow cytometry the expression of CD86, PDL1, ICOSL, and OX40L on DCs cultured for 48 h with TSLP, LPS, or untreated.

We observed that TSLP-DC expressed high levels of ICOSL (Fig. 8, A and B), a molecule important in Tfh polarization (Choi et al., 2011). To assess the role of ICOSL, we

cultured TSLP-DC with CD4 T cells in the presence of an anti-ICOSL blocking antibody and measured cytokines after 6 d. ICOSL blocking did not affect IL-3 or IL-10 levels, and more importantly, did not inhibit polarization by TSLP-DC into Th cells secreting IL-21 and CXCL13 (Fig. 8 C). As a control of the functional blocking of the ICOSL antibody, we detected a decrease in IL-10 production by naive CD4 T cells cultured with plasmacytoid DCs activated with CpGB (pDCs; Fig. 9 A), as previously reported (Ito et al., 2007).

We confirmed OX40L as being induced by TSLP in comparison to DCs or LPS-DC (Fig. 8 A and quantification in Fig. 8 B; Ito et al., 2005). Given the controversial role of OX40L in mouse Tfh development (Deenick et al., 2011), and a recent study on the role of OX40L in human Tfh polarization (Jacquemin et al., 2015), we investigated its role in TSLP-DC-induced Tfh polarization. We used an anti-OX40L blocking antibody during the TSLP-DC CD4 T cell co-culture. OX40L blocking inhibited IL-3 secretion, whereas enhancing IL-10 expression (Fig. 8 D), as previously



**Figure 9. OX40L blocking reduces BCL6 induction by TSLP-DC.** (A) Quantification of IL-10 production using CBA by CD4 T cells differentiated during 6 d with pDC activated with CpGB (15  $\mu$ g/ml during 24 h). Anti-ICOSL blocking antibody or isotype control antibody (25  $\mu$ g/ml) were added at the beginning of the culture. Mean  $\pm$  SEM for six experiments is plotted. \*,  $P < 0.05$  Wilcoxon matched pair test. (B) Quantification by FACS analysis of the percentage of CXCR5<sup>hi</sup>PD1<sup>hi</sup>, CXCR5<sup>lo</sup>PD1<sup>lo</sup> cells in TSLP-DC co-culture at day 4, treated with functional blocking antibodies or isotype controls as indicated. The percentage of each gate is shown. For BCL6 expression, gray histograms represent the FMO signal, and red histograms represent specific BCL6 staining. MFI of specific staining and percentage of BCL6<sup>+</sup> cells are plotted for one representative experiment. (C and D) Quantification as in B, from six independent experiments. SMFI for BCL6 was calculated by subtracting the FMO from BCL6-specific staining in CXCR5<sup>hi</sup>PD1<sup>hi</sup> and CXCR5<sup>lo</sup>PD1<sup>lo</sup> cells. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , paired Student's *t* test.

reported (Ito et al., 2005). We found that OX40L inhibition significantly decreased both CXCL13 and IL-21 secretion by CD4 T cells polarized by TSLP-DC (Fig. 8 D). We investigated the effect of ICOSL and OX40L functional blocking on the expression of CXCR5, PD1, and BCL6. We measured the percentage of CXCR5<sup>hi</sup>PD1<sup>hi</sup> and CXCR5<sup>lo</sup>PD1<sup>lo</sup> cells, and their respective expression of BCL6 in the presence of blocking antibodies and isotype controls, after 4 d of co-culture with TSLP-DC. ICOSL functional blocking increased BCL6 expression by CXCR5<sup>hi</sup>PD1<sup>hi</sup> cells compared with the isotype control (Fig. 9 B, and quantification in D). OX40L functional blocking decreased the percentage of CXCR5<sup>lo</sup>PD1<sup>lo</sup> cells, paralleled by an increase in the percentage of CXCR5<sup>hi</sup>PD1<sup>hi</sup> cells (Fig. 9 C). In line with no significant changes in IL-21 and CXCL13 expression (Fig. 8 D), we could not observe any decrease of BCL6 expression in response to ICOSL functional blocking (Fig. 9 D). However, we observed that OX40L functional blocking induced a significant decrease of BCL6 expression in both CXCR5<sup>lo</sup>PD1<sup>lo</sup> and CXCR5<sup>hi</sup>PD1<sup>hi</sup> cells polarized by TSLP-DC (Fig. 9 D).

In summary, our data demonstrated that TSLP induced Tfh polarization through OX40L, and that OX40L controlled BCL6 expression.

### In vivo evaluation of Tfh markers in atopic dermatitis (AD) and Netherton syndrome (NS) patients

We sought to assess the relevance of the TSLP-DC-polarized Tfh cells in human pathology. AD is a skin allergic pathology characterized by Th2 environments (Brandt and Sivaprasad, 2011), and the role of TSLP in the pathogenesis of AD is well established (Ziegler and Artis, 2010).

We first asked whether Tfh were infiltrating the lesional skin of AD patients. By immunofluorescence, we could not detect CXCL13<sup>+</sup> cells in frozen AD skin sections (Fig. S3A). By FACS, we identified very low percentages (<0.5%) of CXCR5<sup>+</sup> CD4<sup>+</sup> cells in T cell emigrated from lesional skin biopsies of 2 AD donors (Fig. S3 B). Lack of significant Tfh cell infiltration of AD skin prompted us to look for circulating Tfh within AD PBMCs. Circulating human Tfh cells comprise a population of IL-4- and IL-21-producing cells that induce IgE switch in B cells (Morita et al., 2011).

We quantified by FACS the percentage of this Tfh subset cells, gated as CD4<sup>+</sup>CD45RO<sup>+</sup>CXCR5<sup>+</sup>CXCR3<sup>-</sup>CCR6<sup>-</sup>, in PBMCs obtained from age- and gender-matched AD and healthy donors. The percentage of Tfh2 was higher in AD donors as compared with healthy donors (64 vs. 30% of CXCR5<sup>+</sup>CD45RA<sup>+</sup>CD4<sup>+</sup> cells). In parallel, we observed a dramatic decrease of CXCR3<sup>+</sup>CCR6<sup>-</sup> cells (Fig. 10 A).

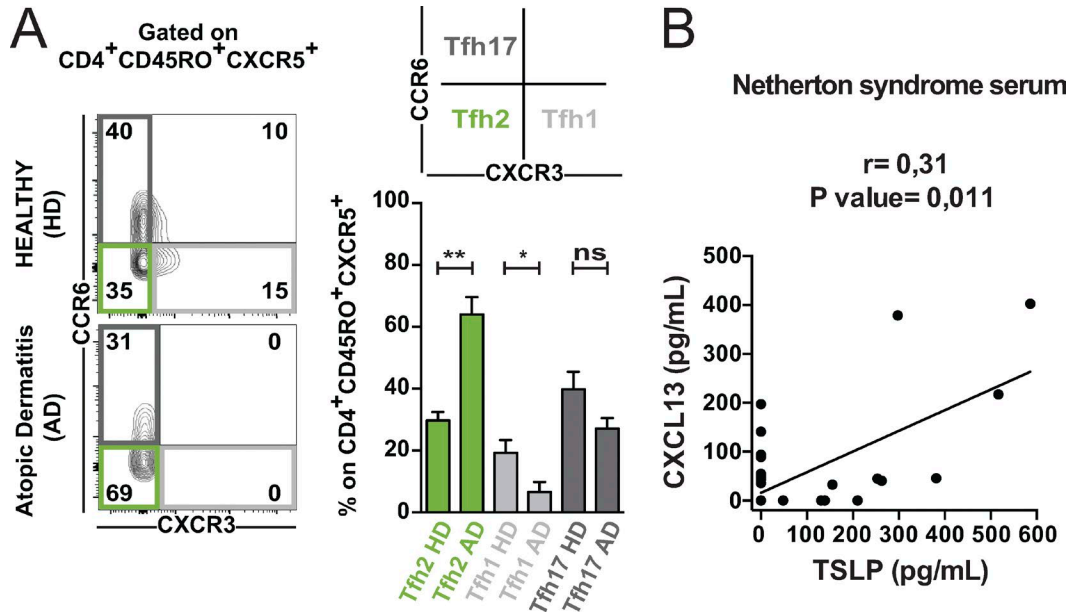


Figure 10. In NS patients, serum TSLP levels positively correlate with CXCL13. (A) FACS analysis showing the frequency of CCR6<sup>-</sup>CXCR3<sup>-</sup> (green), CCR6<sup>-</sup>CXCR3<sup>+</sup>, and CCR6<sup>+</sup>CXCR3<sup>-</sup> populations in the CD4<sup>+</sup>CD45RO<sup>+</sup>CXCR5<sup>+</sup> gate. Representative plots are shown for a healthy donor and AD donor, respectively. Frequency distribution in six AD donors, and four healthy donors are plotted. \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001, paired Student's *t* test. (B) Linear correlation between serum TSLP and CXCL13, measured by ELISA, is shown. Spearman *r* and P-values are plotted. 64 samples from 13 NS patients are plotted.

In addition to AD, which includes predominantly local inflammatory manifestations, we looked for a systemic disease in which TSLP is expressed. This is the case of NS, a rare genetic skin disease characterized by a severe skin barrier defect, atopic manifestations, and elevated IgE levels (Hovnanian, 2013). It has recently been shown that TSLP is highly expressed in a mouse model for NS and in the skin of NS patients (Briot et al., 2009). We analyzed the levels of TSLP in 64 serum samples obtained from 13 NS patients by ELISA. In parallel, we measured CXCL13 as a Tfh marker in the same samples. We found a significant positive correlation between TSLP and CXCL13 in the sera of NS patients (Fig. 10 B).

Collectively, AD and NS patient samples suggest that TSLP and Tfh might be linked in humans in vivo.

**DISCUSSION**

In this study, we provide definitive evidence for a key role of TSLP-activated DCs in the differentiation of naive CD4 T cells into cells possessing Tfh characteristics through the co-stimulatory molecule OX40L.

IL-12, the main driver of Th1 polarization, promotes Tfh differentiation in humans (Schmitt et al., 2009, 2013; Ma et al., 2012). It has been recently shown that the Th17-inducing cytokines IL-23 and TGF-β could trigger Tfh differentiation too (Schmitt et al., 2014). However, Tfh cells are also present in Th2-dominated environments (Glatman Zaretsky et al., 2009; Yusuf et al., 2010; Liang et al., 2011; Kemeny, 2012), and may have an important physiopathological role in mouse models of airway hyperresponsiveness (Coquet et al., 2015;

Ballesteros-Tato et al., 2016). However, how Tfh differentiation can occur in such Th2 environments is not known. The cytokine TSLP was until now associated with human Th2 polarization (Liu et al., 2007; Ziegler and Artis, 2010). Here, we show a novel function of TSLP as the driver of the differentiation of Tfh cells expressing CXCR5, IL-21, CXCL13, BCL6, and helping memory B cells to produce IgG and IgE. How to reconcile the induction of Tfh cell differentiation in a Th2 context, and the reported negative role of IL-4 on human Tfh development (Schmitt et al., 2014), must still be answered. In our data, we observed a co-occurrence of IL-21- and IL-4-producing T cells in TSLP-DC-polarized cultures. However, TSLP-DC do not produce IL-4 (Soumelis et al., 2002), and TSLP-DC-activated T cells start secreting IL-4 around day 4 (Leyva-Castillo et al., 2013), when we could already identify the CXCR5<sup>hi</sup>PD1<sup>hi</sup> population of cells expressing Tfh markers. Therefore, there is an IL-4-free window for Tfh differentiation during the first 48 h of culture, a time when the decision making about Tfh differentiation likely occurs (Choi et al., 2011). Importantly, IL-4 inhibits IL-21 secretion in DC-free settings (Schmitt et al., 2014), different from our DC/T cell co-cultures. We cannot exclude the possibility that, in the context of TSLP-DC-driven Tfh polarization, IL-4 might not inhibit IL-21 production.

The relationship and plasticity between Tfh and Th subsets are still debated. Here, we show that TSLP-DC-induced CXCR5<sup>hi</sup>PD1<sup>hi</sup>IL-21<sup>+</sup>IL-4<sup>+</sup> cells coexpressed the Th2 transcription factor GATA3 and the Tfh transcription factor BCL6. Our data suggest that, in TSLP-DC-activated cells,

GATA3 drives IL-4 expression in the presence of BCL6. In contrast, previous observations showed that BCL6 represses GATA3 in GC Tfh (Kusam et al., 2003; Hatzi et al., 2015). From our data, we could not elucidate the mechanisms underlying GATA3 and BCL6 coexpression. However, our cellular system, based on human primary cells, represents a unique tool to understand the relationship and plasticity between Th2 and Tfh in humans.

Co-stimulatory molecules, in particular ICOS–ICOSL interactions, were shown to be important in Tfh cell development (Choi et al., 2011; Crotty, 2014). The role of other co-stimulatory molecules, and in particular OX40L, is controversial and seems to depend on the experimental mouse model used (Deenick et al., 2011). A recent work shows that OX40L promotes human Tfh responses, particularly in Lupus (Jacquemin et al., 2015). In our work, by using functional blocking of co-stimulatory molecules, we established that OX40L, and not ICOSL, is the main driver of IL-21, CXCL13, and BCL6 expression in T cells by TSLP-DC. Nonetheless, as OX40L functional blocking did not completely abolish IL-21 and CXCL13 production, we cannot exclude that other factors may contribute to the induction of Tfh differentiation by TSLP-DC.

We show that TSLP-DC not only stimulated naive CD4 T cells to acquire Tfh markers, but strongly induced IL-21 and CXCL13 secretion by memory circulating Tfh cells. Additionally, TSLP-DC induced the expression of Tfh markers ICOS, PD1, CXCR5 and BCL6 in memory non Tfh (CXCR5<sup>-</sup>) cells. This result is particularly relevant to Tfh biology because, to our knowledge, this is the first report of reprogramming of human memory non-Tfh CD4 T cells into Tfh-like cells. The frequency of memory circulating Tfh and their activation states have been linked to antibody responses in human subjects (He et al., 2013; Locci et al., 2013). Therefore, it has been proposed that boosting memory Tfh responses could improve vaccine efficacy (Ma and Deenick, 2014). Our study, in combination with published data on the effect of TSLP on mouse antibody responses (Van Roey et al., 2012), provides the rationale to further explore TSLP as a vaccine adjuvant in humans. Additionally, our findings suggest that TSLP, which is produced by epithelial cells, could activate memory Tfh cells in inflamed peripheral tissues through DCs.

How CXCR5<sup>hi</sup>PD1<sup>hi</sup> CD4 cells induced by TSLP-DC relate to reported Tfh subsets is of major importance. We directly compared CXCR5<sup>hi</sup>PD1<sup>hi</sup> cells polarized by TSLP-DC to human tonsillar Tfh (Kim et al., 2004; Bryant et al., 2007; Weinstein et al., 2014). Our data show that the expression profile of key Tfh markers (PD1, CXCR5, ICOS, BTLA, SAP, CD200, CXCL13, IL-21, C-MAF, BCL6, and BLIMP1) by TSLP-DC-induced CXCR5<sup>hi</sup>PD1<sup>hi</sup> cells was similar to tonsillar Tfh and GC Tfh cells.

A characteristic of the IL-21<sup>+</sup> cells we identified, distinguishing them from previously reported Tfh subsets, is the co-production of TNF. 20% of CD4 activated by TSLP-DC coexpressed IL-21 and TNF. We propose that IL-21<sup>+</sup>TNF<sup>+</sup> cells may correspond to a distinct inflammatory Tfh cell

subset. In addition, we also detected cells producing IL-21, but not IL-4, TNF, or IFN- $\gamma$  (16% of IL-21 producers), IL-21<sup>+</sup>IL-4<sup>+</sup> (3% of IL-21 producers), and IL-21<sup>+</sup>IL-4<sup>+</sup>TNF<sup>+</sup> (11% of IL-21 producers). This reveals that TSLP induced a large diversity of Th cells, with potential diverse functions depending on the physiopathological contexts. We observed the co-induction of IFN- $\gamma$ <sup>+</sup> cells, a hallmark of Th1 cells, together with Th2 effector cells. This co-induction reproduces the coexistence of Th1 and Th2 cells in AD (Grewe et al., 1998), where TSLP plays a role in T cell polarization (Ziegler and Artis, 2010).

By co-culturing CXCR5<sup>hi</sup>PD1<sup>hi</sup> and CXCR5<sup>lo</sup>PD1<sup>lo</sup> cells polarized by TSLP-DC with memory B cells, we showed that CXCR5<sup>hi</sup>PD1<sup>hi</sup> cells selectively induced IgE secretion. Therefore, in addition to Tfh markers, cells polarized by TSLP-DC presented Tfh2 functional features (Ueno et al., 2015). We found that IgE secretion was accompanied by IgG4 production. Both IgE and IgG4 have been linked to allergic disorders in humans (Gould et al., 2003). Mechanistically, using anti-IL-4R $\beta$  functional blocking antibody, we showed that IgE induction depended on IL-4 and/or IL-13. Therefore, we described a pathway linking TSLP to IgE production, and involving interactions between epithelial cells, DCs, T cells and B cells.

TSLP is expressed in a broad spectrum of diseases. This is the case of AD (Soumelis et al., 2002), psoriasis (Volpe et al., 2014), NS (Hovnanian, 2013; Furio and Hovnanian, 2014), keloid (Shin et al., 2016), and helminthic infections (Ramalingam et al., 2009; Ziegler and Artis, 2010; Giacomini et al., 2012). In some of these diseases, Tfh cells have been reported (Glatman Zaretsky et al., 2009; Niu et al., 2015). Our analysis on AD clinical samples show that there is an enrichment of Tfh2 and a decrease of Tfh1 in the circulation. A decrease in Th1 cells in PMBC of chronic AD patients has been previously shown (Nakazawa et al., 1997; Lonati et al., 1999). In NS serum samples, we found a positive correlation between TSLP and the GC activity marker CXCL13.

Collectively, our study provides the rationale to exploit TSLP as a pharmacological target to manipulate Tfh polarization in allergic and inflammatory disorders. Acting on an upstream inducer mechanism of Tfh and Tfh2 differentiation may result in additional clinical benefit in the complex pathogenicity of allergy.

## MATERIALS AND METHODS

### Cell purification

Buffy coats were obtained from healthy adult blood donors (Etablissement Français du Sang, Paris, France) in conformity with Institut Curie ethical guidelines. Human blood primary DCs were purified according to an established protocol (Alculumbre and Pattarini, 2016). In brief, after FICOLL (GE Healthcare) gradient centrifugation, total PBMCs were enriched in DCs using the EasySep Human Pan-DC Pre-Enrichment kit (StemCell Technologies). Enriched DCs were sorted to obtain 98% purity on a FACSVantage (Miltenyi

Biotec), as Lineage (CD3, CD14, CD16, and CD19)<sup>-</sup> CD4<sup>+</sup> (Beckman Coulter), CD11c<sup>+</sup> (BioLegend), whereas pDCs were sorted as Lineage<sup>-</sup> CD4<sup>+</sup> CD11c<sup>-</sup>. When detailed, DCs were further separated into subsets by FACS sorting using anti CD1c (eBioscience) and CD141 (Miltenyi Biotec) staining. After enrichment from total PBMCs using the CD4<sup>+</sup> T cell isolation kit (Miltenyi Biotec), naive and memory CD4 T cells were sorted on a FACSARIA (BD) as CD4<sup>+</sup>, CD25<sup>-</sup>, and CD45RA<sup>+</sup> and CD45RO<sup>+</sup>, respectively (BD). Blood Tfh were sorted as CD4<sup>+</sup>CD25<sup>-</sup>CD45RO<sup>+</sup>CXCR5<sup>+</sup> (R&D Systems). Human tonsils were obtained from the Necker Hospital (Paris, France) in conformity with Institut Curie ethical guidelines. Tonsillar CD4 T cells were purified from human tonsils by mechanical disruption (C tube and gentleMACS, Miltenyi), followed by a FICOLL gradient centrifugation. For FACS analysis, total cells were analyzed. For PCR analysis, tonsillar Tfh were enriched using a CD4<sup>+</sup> T cell isolation kit (Miltenyi) and then sorted as CD4<sup>+</sup>, CD19<sup>-</sup>, CD45RO<sup>+</sup>, CXCR5<sup>hi/lo/-</sup>, and PD1<sup>hi/lo/-</sup> (BioLegend) on a FACSARIA (BD).

#### DC and pDC activation

DC and pDC were cultured in RPMI 1640 Medium GlutaMAX (Life Technologies) containing 10% Fetal Calf Serum (Hyclone), 100 U/ml Penicillin/Streptomycin (Gibco), MEM Non-Essential Amino Acids (Gibco), and 1 mM NA pyruvate (GIBCO). DCs were cultured at 10<sup>6</sup>/ml in flat bottom plates for 24 h in the presence of 50 ng/ml rhTSLP where not differently specified (R&D Systems) or 100 ng/ml ultrapure LPS (InvivoGen).

pDCs were cultured at 10<sup>6</sup>/ml in flat-bottom plates for 24 h in the presence of 15 µg/ml CpGBODN2006 (InvivoGen).

#### DC/T co-culture

For co-culture, DCs were washed twice in PBS and put in culture with allogeneic either naive or memory CD4 (10<sup>4</sup> DCs and 5 × 10<sup>4</sup> T cells) in X-VIVO 15 medium (LONZA) for the indicated time. For co-culture, pDC were washed twice and put in culture with allogeneic naive CD4 cells (10<sup>4</sup> pDC and 5 × 10<sup>4</sup> T cells) in Yssel's medium for 6 d. For co-culture, CD4 T cells were freshly purified from PBMC the day after DC purification. Each co-culture experiment was performed by coupling exclusively a single DC donor with a single CD4 T cell donor.

For blocking experiments, DCs or pDCs were incubated at 37°C with 50 µg/ml anti-human OX40L antibody (clone ik-5; provided by T. Hori, Ritsumeikan University, Japan), 25 µg/ml anti-human ICOSL (clone MIH-12; eBioscience), or matched isotype controls (R&D Systems and eBioscience). After 60 min, CD4 naive T cells were added to the culture. Antibodies were maintained for the duration of the co-culture.

At indicated time points, cells were either FACS sorted or used for surface or intracellular staining, or washed and reseeded at 10<sup>6</sup>/ml and treated with anti-CD3/CD28 beads (LifeTech) for 24 h, after which supernatants and cells were collected for analysis.

For primary and secondary co-cultures, CD4 naive T cells were co-cultured with DCs as described at the beginning of this section. At day 5, cells were counted and divided. One part was analyzed for intracellular cytokine production; the other part was put in a secondary culture in the absence of any DCs, in the presence of TSLP-DC or LPS-DC (24 h activation), at the ratio 1:5 in X-VIVO 15 medium. DCs used in the secondary co-culture were purified from donors independent from the DC donors of the primary co-culture and the CD4 T cell donors. Cells were kept in culture for 6 d, and half of the medium was replaced at day 5 with fresh medium.

#### DC-free Th cell polarization

Sorted naive CD4 T cells were cultured with anti CD3/CD28 beads to obtain Th0 or beads plus IL-1β, IL-23, TGF-β, and IL-6 (PeproTech) to obtain Th17 as already published (Volpe et al., 2008) for 5 d. When indicated, 50 ng/ml TSLP was added at the beginning of the culture, and cells were cultured for 6 d. At the end of the culture, cells were washed, reseeded at 10<sup>6</sup>/ml, and treated with anti-CD3/CD28 beads; supernatants and cells were collected for analysis after 24 h.

#### T/B co-culture

After 4 d of co-culture with TSLP-DC, activated CD4 T cells were FACS sorted as CXCR5<sup>hi</sup>/PD1<sup>hi</sup> or CXCR5<sup>lo</sup>/PD1<sup>lo</sup>. The same day, autologous PBMC were thawed and, after a round of human memory B cell Enrichment (Miltenyi Biotec), memory B cells were FACS sorted as CD3<sup>-</sup>CD19<sup>+</sup>CD27<sup>+</sup>IgD<sup>-</sup> cells. T and B cells were co-cultured in X-VIVO medium in round-bottom plates (2.5 × 10<sup>5</sup> T and 2.5 × 10<sup>5</sup> memory B). Memory B cells alone were cultured with 1 µg/ml rhCD40L (Alexis) and 2.5 µg/ml CpG B or left untreated. At day 14 of culture, cells were harvested for flow cytometry analysis and supernatants stored at -80°C to quantify Igs.

For IL4R-α functional blocking, sorted CXCR5<sup>hi</sup>/PD1<sup>hi</sup> or CXCR5<sup>lo</sup>/PD1<sup>lo</sup> cells were incubated at 37°C with 20 µg/ml of anti-IL4R-α or IgG2a isotype control (R&D Systems). After 1 h, autologous-sorted memory B cells were added (2.5 × 10<sup>5</sup> T cells and 2.5 × 10<sup>5</sup> memory B cells). Supernatants were recovered after 14 d of co-culture, stored at -80°C for IgG and IgE measurement by cytometric bead array (CBA).

#### Flow cytometry analysis

Antibodies and matched isotypes were titrated on the relevant human PBMC population. For surface FACS analysis, the antibodies recognizing these proteins were used: PDL1 (BD), CD86 (BD), OX40L (Ancell), ICOSL (R&D Systems), ICOS (eBioscience), PD1 (BD), CXCR5 (R&D Systems or BD), BTLA (BioLegend), CD200 (eBioscience), CCR7 (BD), TSLPR (BioLegend), IL7Ra (eBioscience), CD27 (BD), and CD38 (Miltenyi Biotec). Dead cells were excluded using DAPI (Miltenyi Biotec).

For intracellular cytokine staining, CD4 T cells were stimulated with 100 ng/ml PMA plus 500 ng/ml Ionomycin.

cin. When cells were sorted before intracellular staining, they were cultured overnight in X-VIVO medium at  $10^6$  cells/ml before PMA and Ionomycin stimulation. After 90 min, 3  $\mu\text{g/ml}$  Brefeldin A (eBioscience) was added and kept for 4 h. To exclude dead cells, CD4 T cells were stained using the LIVE/DEAD Fixable yellow dead cell stain kit, following manufacturer's instructions (LifeTech). Cells were fixed and permeabilized using the IC Fix and Permeabilization buffers (eBioscience). Intracellular cytokines were revealed with fluorescently conjugated antibodies against IL-21 (BD), TNF (BioLegend), IL-4, and IFN- $\gamma$ , or matched isotype controls (eBioscience) and acquired on a LSR Fortessa instrument (BD).

For transcription factor intracellular staining, dead cells were first stained with a Zombie-NIR dye (BioLegend), followed by PD1 and CXCR5 (BD) staining. After fixation and permeabilization using the FOXP3 IC buffer kit (eBioscience), cells were stained with an anti-BCL6 antibody (BD), TBET, GATA3, RORC, C-MAF, or SAP (eBioscience) and acquired on a LSR Fortessa instrument. As a control for intracellular staining of transcription factors, cells were stained using PD1, CXCR5, and CD4 (to define the populations) and matched isotype controls at the same concentration as the transcription factor antibodies. The fluorescence obtained in each channel and in each population in the presence of isotype control antibody (Fluorescence minus one [FMO]) was subtracted from the fluorescence obtained by the specific staining of transcription factors in each population. Sorted naive CD4 T cells were analyzed in parallel as a control.

### Flow cytometry data processing

FACS data were analyzed using the FlowJo software (Tree Star).

### Cytokine quantification

Cytokines were quantified in the supernatants using ELISA for IL-21 (BioLegend) and CXCL13 (R&D Systems) or CBA flex set for IL-3, IL-4, IL-5, IL-10, IL-13, IL-17A, TNF, and IFN- $\gamma$  (BD), following the manufacturer's protocol. Total human IgG, IgE, IgG4, and IgM were quantified using the Human IgGs Flex Sets (BD).

### PCR

Cells were sorted and lysed in RLT buffer. RNA extraction was performed using the RNeasy micro kit (QIAGEN) according to manufacturer's instructions. Total RNA was retrotranscribed using the superscript II polymerase (Invitrogen) in combination with random hexamers, oligo dT, and dNTPs (Promega).

Transcripts were quantified by real time PCR on a 480 LightCycler instrument (Roche). Reactions were performed in 10  $\mu\text{l}$ , using a master mix (Eurogentec), with the following TaqMan Assays (all from Life Technologies): BCL6 (Hs00153368\_m1), PRMD1 (Hs00153357\_m1), BTLA (Hs00699198\_m1), CXCR4 (Hs00607978\_s1), CXCR5 (Hs00540548\_s1), CXCL13 (Hs00757930\_m1), ICOS (Hs00359999\_m1), IL-21 (Hs00222327\_m1), PDCD1 (Hs01550088\_m1), SH2D1A (Hs00158978\_m1), CCR7 (Hs00171054\_m1), CD200 (Hs01033303\_m1), IL-4 (Hs00174122\_m1), TNF (Hs00174128\_m1), MAF (Hs00193519\_m1), GATA-3 (Hs00231122\_m1), TBX-21 (Hs00203436\_m1), RORC (Hs01076112\_m1), FOXP3 (Hs00203958\_m1), IL-5 (Hs00174200\_m1), IL-13 (Hs99999038\_m1), IFNG (Hs00174143\_m1), and IL-17A (Hs00174383\_m1). Crossing points (Cp) from each analyte were obtained using the second derivative maximum method, and the transcripts were quantified as fold changes in comparison to the mean of the two housekeeping genes (B2M [Hs99999907\_m1] and RPL34 [Hs00241560\_m1]).

### Analysis of AD and HD PBMCs

After obtaining informed consent from patients, whole blood was taken from AD patients ( $n = 6$ , Table 1). PBMCs were purified using CPT tubes (BD) and immediately frozen. Local ethics committees of the Heinrich-Heine University (Düsseldorf, Germany) approved the study. Healthy age- and gender-matched controls were also included in the study, and were processed as AD samples at the Heinrich-Heine University.

Total PBMCs from healthy donors and from AD patients ( $5 \times 10^6$  each), were thawed and immediately stained for sorting. Cells were stained using CD4 (BD), CD45RO (BD), CXCR5 (R&D Systems), CXCR3 (BD), and CCR6 (BioLegend) for 30 min at  $37^\circ\text{C}$ .

Table 1. **Clinical data**

Patient no.	Gender	Year of birth	Diseases	SCORAD
1	M	1975	AD	41
2	W	1957	AD	41
3	M	1962	AD	39
4	W	1997	AD	35.4
5	W	1998	AD	38.2
6	W	1989	AD	44.4
7	M	1980	HD	
8	W	1970	HD	
9	W	1987	HD	
10	W	1969	HD	

M, man; W, woman. SCORAD (Scoring of AD) was assessed following the Consensus report of the European task force on AD.

## Immunofluorescence

Frozen tissue slides (human tonsils and skin) were stained with rat anti-human TSLP (clone 12F3; gift from L. Bover, MD Anderson Cancer Center, Houston, Texas), goat anti-human CXCL13 (R&D Systems), followed by incubation with fluorescence-conjugated secondary antibodies. Slides were stained with DAPI, mounted with Vectashield (Vector) and acquired using an Eclipse microscope (Nikon).

## Cell purification from human skin

Fresh AD lesional skin biopsies were washed in PBS, minced with a scalpel, and placed in culture at 37°C with 5% CO<sub>2</sub> in RPMI 1640 complemented with 2 mmol/liter glutamine, 1 mmol/liter sodium pyruvate, 1% nonessential amino acids, 0.05 mmol/liter 2-mercaptoethanol, 100 U/ml penicillin, and 100 µg/ml streptomycin (Lonza) with 5% autologous plasma and 60 U/ml recombinant human IL-2 (Novartis) to obtain enriched skin T cells. Medium was replaced every third day, and after 8 to 10 d, T cells that emigrated from tissue samples were collected and placed in starvation with low IL-2 before phenotypic characterization.

## Statistical analysis

Statistical analysis was performed using the Prism software v7 (GraphPad). Paired Wilcoxon or *t* test were applied as detailed to compare two groups. Mann-Whitney test was used for nonpaired analysis. Significance was retained for *P* < 0.05.

qPCR data were normalized and center reduced using Box-Cox transformation, and plotted using heat map package in the R software.

## Online supplemental material

Fig. S1 shows that human naive CD4 T cells do not express TSLPR, and do not respond to TSLP stimulation by expressing IL-21. Fig. S2 details the expression of Tfh and Th markers shown in Fig. 4. Fig. S3 displays the expression of CXCL13 and CXCR5 in AD and healthy donor skin samples, by IHC and FACS staining, respectively.

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Author contributions: L.P. designed, performed and analyzed experiments and wrote the manuscript, C.T. performed experiments, analyzed data and prepared Figures, S.B. performed experiments, M.G. performed intracellular staining, M.D. purified tonsillar Tfh and performed PCR, T.H. provided the anti-OX40L ab. V.S. supervised the study.

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