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OUTSTANDING OBSERVATION

Early commitment of naïve human CD4$^+$ T cells to the T follicular helper (T_{FH}) cell lineage is induced by IL-12

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T follicular helper (T_{FH}) cells are a specialized subset of CD4$^+$ T cells that localize to B-cell follicles, where they are positioned to provide help for the induction of optimal humoral immune responses. Key features of T_{FH} cells are the expressions of CXCR5, ICOS, interleukin (IL)-21 and *BCL-6*. The requirements for human T_{FH} cell development are unknown. Here we show that IL-6, IL-12, IL-21 and IL-23 are capable of inducing IL-21 expression in naïve CD4$^+$ T cells isolated from human tonsils, peripheral blood and cord blood. However, only IL-12 induced sustained expressions of CXCR5 and ICOS on these activated naïve CD4$^+$ T cells, and endowed them with the ability to provide increased help to B cells for their differentiation into immunoglobulin-secreting cells. The effects of IL-12 were independent of interferon-γ and T-bet, and associated with upregulation of *BCL-6* expression. Thus, these cytokines, particularly IL-12, are likely to act at an early stage during dendritic cell-mediated priming of naïve CD4$^+$ T cells into a T_{FH} cell fate, and thus underpin antibody-mediated immunity.
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The differentiation of naïve CD4$^+$ T cells into Th1, Th2, Th17 and T regulatory cell (Treg) lineages requires the co-ordinated action of cytokines provided by the stimulatory microenvironment, which activate specific transcriptional programs to induce the expression of a set of molecules responsible for the effector function of these CD4$^+$ T cell populations. Thus, interleukin (IL)-12, IL-4 and transforming growth factor (TGF)-β guide the differentiation of naïve CD4$^+$ T cells into Th1, Th2 and Treg cells, respectively, whereas the combinations of TGF-β–IL-6 or TGF-β–IL-21 and IL-1β–IL-6–IL-23 with or without TGF-β induce murine and human Th17 cells from naïve precursors.[1,2] These cytokines act by inducing expression of lineage-specific transcription factors, such as STAT4–T-bet, STAT6–GATA3, STAT3–RORγt and Foxp3, which are also required for CD4$^+$ T cells to commit to Th1, Th2, Th17 and Treg fates, respectively.[1–3]

Another important population of effector CD4$^+$ T cells is T follicular helper (T_{FH}) cells. The original studies of T_{FH} cells reported that they (a) could be identified by expression of the B-cell zone homing chemokine receptor CXCR5, (b) localized to the B-cell follicle of secondary lymphoid tissues with some being positioned within germinal centres (GC) and (c) were capable of providing help to B cells for their differentiation into memory cells and plasma cells.[4–6] Subsequent microarray analyses extended these findings, by demonstrating that T_{FH} cells exhibited a gene expression profile distinct from Th1 and Th2-type cells, with them expressing high levels of genes encoding the cytokine IL-21, the transcription factor *BCL-6* and several surface molecules including ICOS and PD-1.[7–10] The molecular requirements for generating murine T_{FH} cells have recently begun to be addressed. On one hand, T_{FH} cells were reduced in mice deficient in IL-6, IL-21, IL-21R and STAT3[10–13] suggesting that, akin to Th17 cells, signals delivered through these STAT3-mediated pathways are critical for T_{FH} cells. However, T_{FH} cells still developed in these mice, albeit at a reduced frequency, suggesting that other factors also contribute to their formation. On the other hand, similar studies found that STAT3-, IL-21R- or IL-21-deficiency did not compromise T_{FH}-cell development.[13,14] Thus, in contrast to other CD4$^+$ T cell subsets, our understanding of the factors involved in generating T_{FH} cells remains incompletely defined. Furthermore, the requirements underlying the development of human T_{FH} cells have not been determined.

Here, we have studied human tonsillar CD4$^+$ T cells to delineate cardinal features of T_{FH} cells, which could then be used to identify soluble factors that induce such features in *in vitro* stimulated naïve CD4$^+$ T cells. We found that several cytokines, such as IL-6, IL-12, IL-21 and IL-23, induced naïve CD4$^+$ T cells to produce

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IL-21, with IL-12 being most efficient. Furthermore, exposure to IL-12, but not other cytokines, resulted in naïve $CD4^+$ T cells acquiring additional characteristics of TFH cells, such as increased expressions of *BCL-6*, CXCR5 and ICOS, and an ability to provide help to B cells for their differentiation into Ig-secreting cells. Thus, IL-12, which is produced by activated dendritic cells (DCs), is likely to be a key cytokine involved in regulating the commitment of naïve $CD4^+$ T-cell precursors to the T_{FH} lineage.

RESULTS

Identification and characterization of primary human T_{FH} cells

It is widely accepted that T_{FH} cells can be defined by the expression of CXCR5.[4–6,15] Although >50% of tonsillar $CD4^+$ T cells express CXCR5, only some of them localize to GCs, which is the likely site of their action. This suggests that the population of $CD4^+CXCR5^+$ T cells is heterogeneous, with only a small subset of them corresponding to bona fide T_{FH} cells. This is supported by previous studies that divided human $CD4^+CXCR5^+$ T cells into subsets according to the differential expression of ICOS[9] or CD57.[6,16] To refine the identification of human T_{FH} cells, we labelled tonsillar $CD4^+$ T cells with CD45RA and CXCR5 monoclonal antibodies (mAbs). This revealed four distinct subsets of $CD4^+$ T cells—$CD45RA^+CXCR5^-$ naïve, and three subsets of $CD45RA^-$ T cells that expressed low, intermediate and high levels of CXCR5 ($CXCR5^{lo}$, $CXCR5^{inter}$, $CXCR5^{hi}$; Figure 1)—which could then be examined for phenotype, and expression of transcription factors and cytokines.

Phenotype: Assessment of expression of a broad array of molecules confirmed that $CD4^+CD45RA^+CXCR5^-$ cells were largely naïve cells, as they were $CCR7^+$ and lacked expression of all activation molecules examined (Figure 1). In contrast, $CXCR5^{hi}$ $CD4^+$ T cells uniformly displayed the highest levels of ICOS, PD-1, CD200, CD57 and SAP (Figure 1), molecules previously reported to be associated with T_{FH} cells, as determined largely by microarray analysis of subsets of tonsillar $CXCR5^+CD4^+$ T cells.[7–9] Extended phenotypic analysis demonstrated that CD40 L, OX40, BTLA, CD69, CD126 and CXCR4 were also highly expressed by $CXCR5^{hi}$ $CD4^+$ T cells, whereas CD127, CXCR3, CCR6 and CCR7 were absent from these cells. $CD45RA^-CXCR5^{lo}$ and $CXCR5^{inter}$ T cells expressed these molecules at levels between those on naïve and $CXCR5^{hi}$ $CD4^+$ T cells. Furthermore, the $CD45RA^-CXCR5^{lo}$ and $CXCR5^{inter}$ T-cell subsets were heterogeneous as they contained sub-populations of cells that were positive and negative for both CXCR3 and CCR6, chemokine receptors associated with Th1 and Th17 lineages, respectively[17,18] (Figure 1). Assessment of the SLAM family of surface receptors, which signal

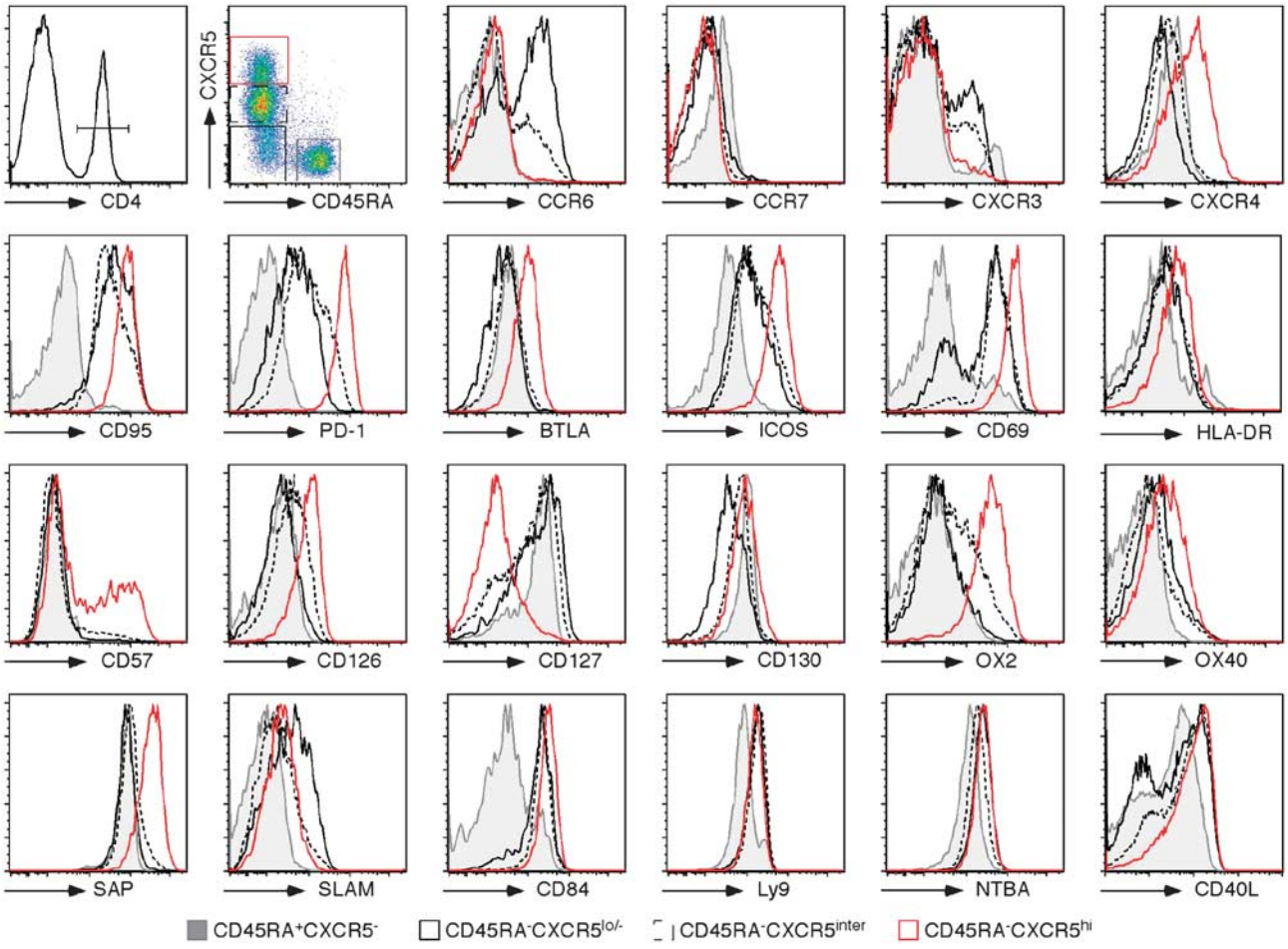

Figure 1 $CD4^+CXCR5^{hi}$ T cells phenotypically resemble terminally differentiated T_{FH} cells. $CD4^+$ tonsillar T cells that were $CD45RA^+CXCR5^-$, $CD45RA^-CXCR5^{lo}$, $CD45RA^-CXCR5^{inter}$ and $CD45RA^-CXCR5^{hi}$ were phenotyped for various cell surface or intracellular molecules known to be expressed on T_{FH} cells and other T-helper subsets. In the instance of CD40L, sorted T-cell populations were cultured with PMA and ionomycin for 2 h to induce expression. The plots are representative of three donor tonsils.

through SAP,[19] demonstrated that $CD4^+CXCR5^{hi}$ T cells expressed high levels of CD84, Ly9 (CD229) and NTB-A, but not SLAM itself. These molecules were also upregulated on $CXCR5^{lo}$ and $CXCR5^{inter}$ $CD4^+$ T cells compared with naïve $CD4^+$ T cells.

Transcription factors: *BCL-6* is highly expressed in $CD4^+CXCR5^+$ T cells[8,9,20] and has recently been demonstrated to be required for the generation of the T_{FH} lineage[21,22] in a manner analogous to T-bet, GATA3, Foxp3 and RORγt for establishing the Th1, Th2, Treg and Th17 lineages, respectively.[15,23] To characterize human tonsillar $CD4^+$ T-cell subsets further, we determined the expression of these transcription factors in $CD45RA^+CXCR5^-$, $CD45RA^-CXCR5^{lo}$, $CD45RA^-CXCR5^{inter}$ and $CD45RA^-CXCR5^{hi}$ populations. We also assessed *PRDM1* (encoding Blimp-1) because Bcl-6 and Blimp-1 functionally antagonize each other to regulate B-cell differentiation,[24] and a similar finding was recently made for murine T_{FH} cells.[22] $CD4^+CXCR5^{hi}$ T cells indeed expressed the highest level of *BCL-6* (Figure 2a). They also had low and variable levels of *TBX21* (encoding T-bet) and *GATA3*, respectively, but essentially lacked *RORC* (encoding RORγt), *FOXP3* and *PRDM1*. In contrast, $CD45RA^-CXCR5^{lo}$ cells expressed the highest levels of *TBX21*, *GATA3*, *RORC*, *FOXP3* and *PRDM1*, and the lowest levels of *BCL-6* (Figure 2a). $CD4^+$ $CD45RA^-CXCR5^{inter}$ T cells expressed *BCL-6* and *GATA3* at levels similar to naïve $CD4^+$ T cells, whereas the expression of *TBX21*, *RORC*, *FOXP3* and *PRDM1* in these cells was intermediate to those expressed by $CXCR5^{lo}$ and $CXCR5^{hi}$ cells (Figure 2a).

Cytokine expression: Another characteristic of T_{FH} cells is their expression of high levels of the cytokine IL-21, again as determined by microarray studies.[8,9,20] Thus, it was important to assess the cytokine profile of tonsillar $CD4^+$ T-cell populations, with respect to the expression of not only IL-21 but also other cytokines. This was achieved by stimulating sort-purified $CD45RA^+CXCR5^-$, $CD45RA^-$ $CXCR5^{lo}$, $CD45RA^-CXCR5^{inter}$ and $CD45RA^-CXCR5^{hi}$ $CD4^+$ T cells with either phorbol myristic acetate (PMA) and ionomycin for 6 h or anti-CD3 and anti-CD28 mAb for five days and then assessing IL-2, IL-4, IL-10, IL-13, IL-17A, IL-21, IL-22 and interferon (IFN)-γ production by intracellular staining or enzyme-linked immunosorbent assay (ELISA), respectively. $CD45RA^+CXCR5^-$ T cells expressed IL-2, but very little of the other cytokines (Figures 2b and c). This was further confirmation of their naïve status. In contrast, $CD45RA^-CXCR5^{lo}$ T cells expressed the greatest amounts of IFN-γ, IL-13, IL-17 and IL-22, and low amounts of IL-4, IL-10 and IL-21 (Figures 2b and c). On the other hand, production of IL-4 and IL-21 increased and that of IL-17A, IL-22 and IFN-γ decreased with the acquisition of CXCR5, such that the $CXCR5^{hi}$ subset contained the greatest frequency of $IL\text{-}4^+$ and $IL\text{-}21^+$ cells, but essentially lacked expression and/or secretion of canonical Th1 (IFN-γ) and Th17 (IL-17, IL-22) inflammatory

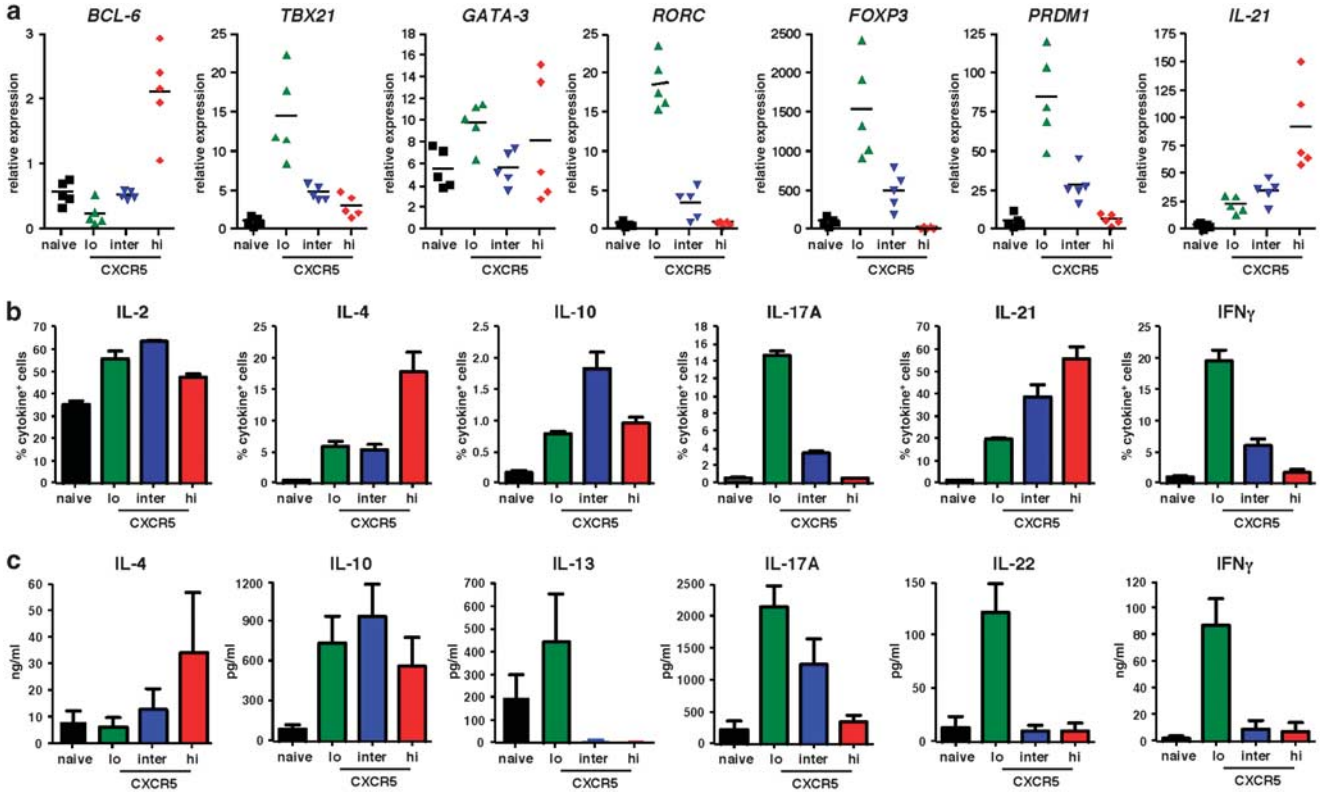

Figure 2 $CD4^+CXCR5^{hi}$ T cells express high levels of *BCL-6* and IL-21. $CD45RA^+CXCR5^-$, $CD45RA^-CXCR5^{lo}$, $CD45RA^-CXCR5^{inter}$ and $CD45RA^-CXCR5^{hi}$ tonsillar $CD4^+$ T-cell populations were isolated by cell sorting. (**a**) RNA was extracted and transcribed into cDNA, which was then used as template for quantitative PCR (q-PCR) to assess expression levels of *BCL-6*, *TBX21, GATA3, RORC, FOXP3, PRDM1* and *IL-21*. Each data point represent results from an individual experiments (n=5) carried out on four separate donor tonsils. (**b**) $CD45RA^+CXCR5^-$, $CD45RA^-CXCR5^{lo}$, $CD45RA^-CXCR5^{inter}$ and $CD45RA^-CXCR5^{hi}$ tonsillar $CD4^+$ T-cell subsets were stimulated with PMA and ionomycin for 6 h and cytokine expression determined by intracellular staining and flow cytometry. The values represent the mean ± s.e.m. of three experiments carried out on different donor tonsils. (**c**) $CD45RA^+CXCR5^-$, $CD45RA^-CXCR5^{lo}$, $CD45RA^-CXCR5^{inter}$ and $CD45RA^-CXCR5^{hi}$ tonsillar $CD4^+$ T-cell subsets were cultured with immobilized anti-CD3 mAb, soluble anti-CD28 and IL-2 for five days and cytokine secretion in culture supernatants was determined by ELISA. The values represent the mean ± s.e.m. of five independent experiments using different donor tonsils.

mediators (Figures 2b and c). Elevated expression of IL-21 by CXCR5^{hi} CD4^{+} T cells was also demonstrated by quantitative PCR (q-PCR) analysis of *ex vivo* isolated CD4^{+} T-cell subsets (Figure 2a). Analysis of co-expression of IL-21 and IFN-γ, IL-17A or IL-4 confirmed these results as the majority of CXCR5^{hi} CD4^{+} T cells that expressed IL-4 co-expressed IL-21, whereas the CD4^{+}CXCR5^{hi} IL-21^{+} cells lacked expression of IFN-γ and IL-17A (Figure 3). Thus, CD45RA^{-}CXCR5^{hi} cells have acquired an IL-21^{hi}IL-4^{hi}IL-10^{+}IL-17A^{-}IL-22^{-}IFN-γ^{-} cytokine profile. In contrast to CXCR5^{hi} CD4^{+} T cells, although CXCR5^{lo} cells express both IL-21 and IL-4 (Figure 2b), single-cell analysis revealed that expression of IL-21 and IL-4 occurred within distinct subsets (Figure 3). On the other hand, CD4^{+}CXCR5^{lo} T cells co-expressing IL-21 together with IFN-γ or IL-17A were clearly detectable.

This analysis allowed us to identify human tonsillar CD4^{+} CXCR5^{hi} T cells as T_{FH} cells, with their signature features being: a unique surface phenotype (ICOS^{hi}CD95^{hi}PD-1^{hi}CD200^{hi}SAP^{hi} CD126^{+}BTLA^{+}OX40^{+}CXCR4^{+} CD127^{-}, Figure 1), high expression of *BCL-6* (coupled with the loss of transcriptional regulators responsible for the generation of other Th cell fates, Figure 2a), and copious production of B-helper cytokines particularly IL-21, IL-4 and IL-10 (Figures 2b and c and Figure 3).

IL-12 modulates the expression of IL-21 by tonsils CD4^{+} T-cell subsets

To investigate the regulation of IL-21 expression in human CD4^{+} T cells, we first sorted subsets of tonsil CD45RA^{+}CXCR5^{-}, CD45RA^{-}CXCR5^{lo} CD45RA^{-}CXCR5^{inter} and CD45RA^{-}CXCR5^{hi} CD4^{+} T cells, and cultured them under various conditions. After five days, cells were re-stimulated with PMA and ionomycin and the frequency of IL-21 producing cells determined. Consistent with *ex vivo* analysis of IL-21 expression, CD45RA^{+}CXCR5^{-} CD4^{+} T cells contained very few (<2%) IL-21^{+} cells after culture under non-polarizing conditions (nil), whereas 20–40% of CD45RA^{-}CXCR5^{lo}, CD45RA^{-}CXCR5^{inter} and CD45RA^{-}CXCR5^{hi} CD4^{+} T cells expressed IL-21 (Figure 4a). Similar to studies carried out in mice,[10,12,25] IL-21 weakly increased the frequency of IL-21^{+} cells from the CD45RA^{+}CXCR5^{-} subset. Although the culture of CD45RA^{+}CXCR5^{-} CD4^{+} T cells under Th2 conditions appeared to inhibit the expression of IL-21 (Figure 4a), culture under Th1 polarizing conditions yielded the greatest frequency of IL-21-expressing cells (Figure 4a), with its effect being much greater than that of IL-21 (that is, sixfold versus twofold increase, over cells cultured under non-polarizing conditions). It is interesting to note that IL-12 had a reduced effect and IL-21 had no effect on IL-21 expression by the activated memory/CD45RA^{-} T cell populations (1.2–2-fold increase), suggesting that as effector CD4^{+} T cells acquire expression of IL-21, they become less sensitive to the modulatory effect of cytokines, such as IL-12 and IL-21.

Induction of IL-21 expression in naïve CD4^{+} T cells—predominant effect of IL-12

Although CD45RA^{+}CD4^{+} T cells isolated from human tonsils are considered to be largely naïve, it is possible that some of them had previously encountered antigen (Ag), or had been exposed to a stimulatory environment *in vivo*. Thus, to examine further the ability of specific cytokines to induce IL-21 expression in CD4^{+} T cells, we used CD45RA^{+}CD4^{+} T cells from cord blood (CB) as a source of Ag-naïve cells. Although the culture of CB-naïve CD4^{+} T cells under

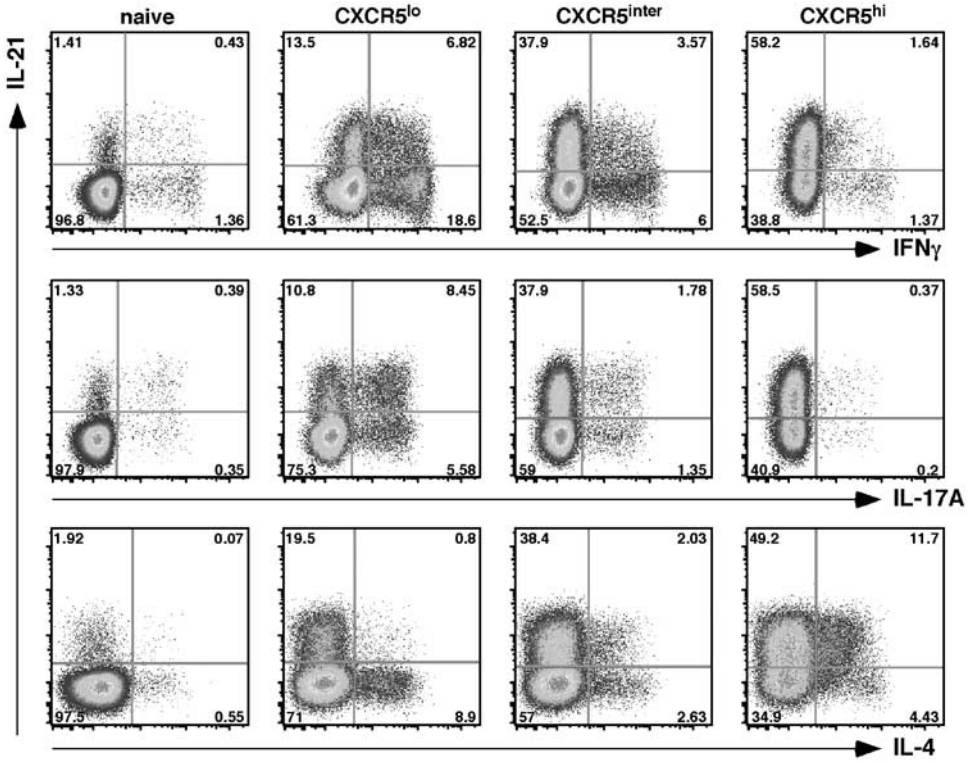

Figure 3 CD4^{+}CXCR5^{hi} T cells co-express IL-21 and IL-4, but not IL-17A and IFN-γ. CD45RA^{+}CXCR5^{-}, CD45RA^{-}CXCR5^{lo}, CD45RA^{-}CXCR5^{inter} and CD45RA^{-}CXCR5^{hi} tonsillar CD4^{+} T-cell populations were isolated by cell sorting, stimulated with PMA and ionomycin for 6 h and then co-expression of IL-21 and either IFN-γ (top row), IL-17A (middle row) or IL-4 (bottom row) was determined by intracellular staining. The dot plots are representative of experiments carried out on three different donor tonsils.

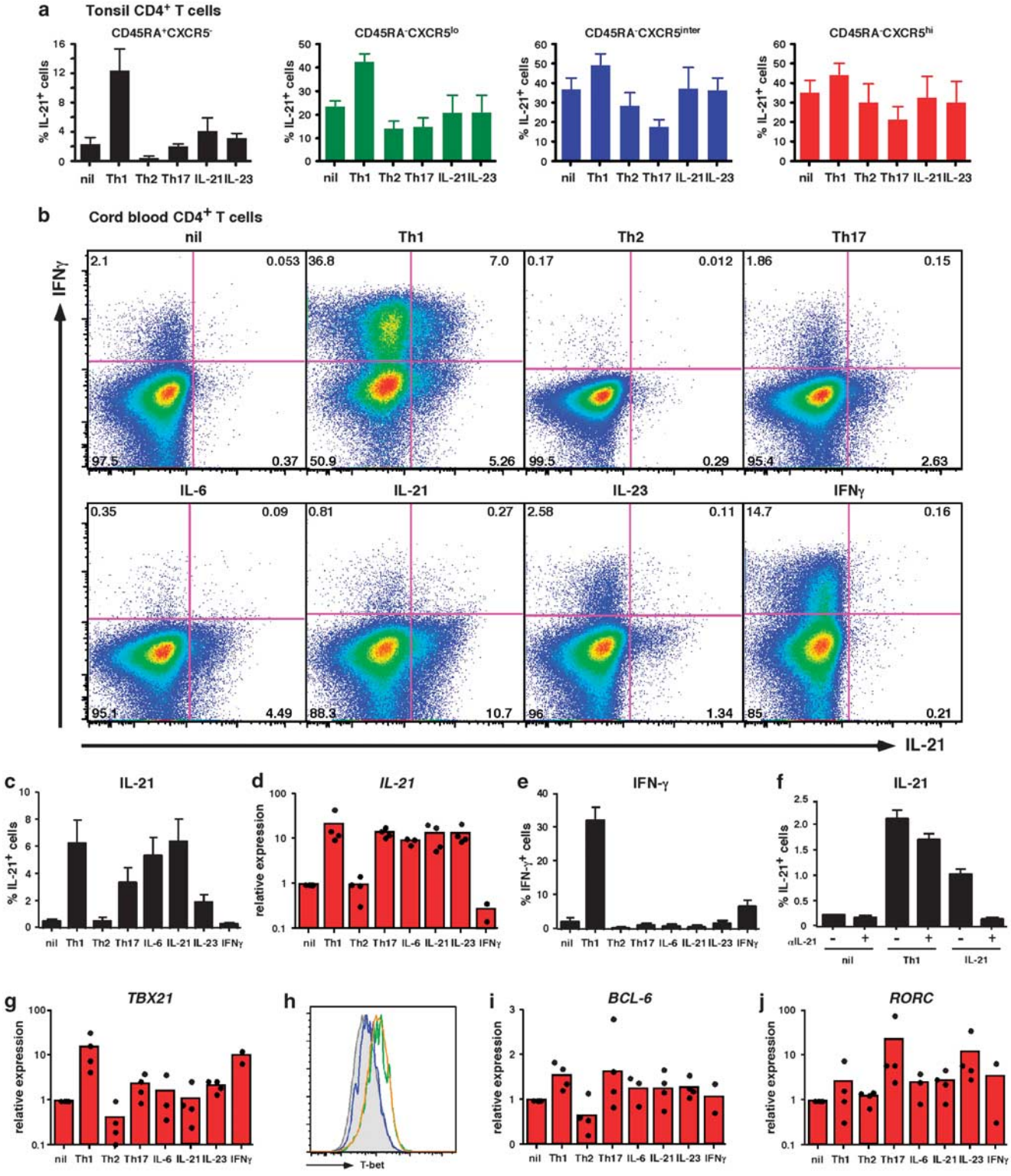

Figure 4 IL-12 induces the generation of IL-21 expressing cells. (**a**) CD45RA$^+$CXCR5$^-$, CD45RA$^-$CXCR5^{lo}, CD45RA$^-$CXCR5^{inter} and CD45RA$^-$CXCR5^{hi} CD4$^+$ T-cell populations were cultured for five days under various polarizing conditions. Cells were then restimulated and intracellular IL-21 expression determined. The values represent the mean ± s.e.m. of 6 independent experiments. (**b**–**j**) CD4$^+$CD45RA$^+$ cord blood T cells were isolated and cultured under various conditions. After 4–5 days cells were re-stimulated with PMA and ionomycin and the frequency of IL-21 (**b**, **c** and **f**), IFN-γ (**b**, **e**), and T-bet (**h**) expressing cells determined, or RNA was isolated and transcribed into cDNA and *IL-21* (**d**), *TBX21* (**g**), *BCL-6* (**i**) and *RORC* (**j**) expression determined by q-PCR. For intracellular cytokine expression, the values represent the mean ± s.e.m. of eight different experiments. For q-PCR analysis, each value is derived from an independent experiment with the bars representing the mean.

Table 1 Induction of IL-21 expression in *in vitro* stimulated human naïve peripheral blood CD4$^+$ T cells

Culture	*%IL-21$^+$*	*%IL-21$^+$IFN-γ$^+$*	*%IL-21$^+$IFN-γ$^-$*	*%IL-21$^-$IFN-γ$^+$*
Nil (*n*=4)	3.0 ± 2.0	0.17 ± 0.1	2.8 ± 1.7	1.0 ± 0.4
Th1 (*n*=4)	18.0 ± 8.0	4.62 ± 2.2	13.3 ± 5.8	13.6 ± 3.3
Th2 (*n*=3)	1.05 ± 0.5	0.02 ± 0.01	1.0 + 0.5	0.34 ± 0.2
Th17 (*n*=4)	2.4 ± 1.4	0.05 ± 0.01	2.4 ± 1.4	0.6 ± 0.2
IL-6 (*n*=4)	4.9 ± 4.3	0.08 ± 0.1	4.8 ± 4.3	0.4 ± 0.2
IL-21 (*n*=4)	6.1 ± 5.5	0.12 ± 0.1	6.0 ± 5.4	0.4 ± 0.2
IL-23 (*n*=4)	4.9 ± 4.4	0.17 ± 0.1	4.8 ± 4.3	1.0 ± 0.4
IFN-γ(*n*=2)	0.2 ± 0.1	0.02 ± 0.01	0.22 ± 0.1	1.1 ± 0.1

Sort-purified peripheral blood CD45RA$^+$CCR7$^+$CXCR5$^-$ CD4$^+$ T cells were cultured for five days under the indicated polarizing conditions or in the presence of the indicated cytokines. After this time, the cells were harvested, re-stimulated with PMA/ionomycin and expression of intracellular IL-21 and IFN-γ determined by flow cytometry. The values represent the mean ± s.e.m. of two to four independent experiments.

non-polarizing or Th2-polarising conditions failed to give rise to IL-21-expressing cells, exposure to IL-12 caused a 5–15-fold increase in the expression of IL-21, as determined by the frequency of IL-21$^+$ cells (Figures 4b and c). IL-21 and, to a lesser extent, IL-6, IL-23 and Th17 culture conditions also induced IL-21 expression (Figures 4b and c). Consistent with the detection of intracellular expression of IL-21, q-PCR analysis further confirmed acquisition of *IL-21* by naïve CB CD4$^+$ T cells cultured under Th1 or Th17 conditions or in the presence of IL-6, IL-21 or IL-23, but not with IL-4 (Th2; Figure 4d). This confirms the ability of IL-12 to induce naïve CD4$^+$ T cells to undergo *de novo* differentiation into IL-21$^+$ cells. To demonstrate that this is not unique to CD4$^+$ T cells isolated from CB, we also assessed the induction of IL-21 in naïve CD4$^+$ T cells isolated from adult peripheral blood (PB). Culture of naïve PB CD4$^+$ T cells under Th1 conditions substantially increased (mean sixfold) the frequency of IL-21$^+$ cells (Table 1). IL-6, IL-21 and IL-23 also increased IL-21 expression by naïve PB CD4$^+$ T cells, albeit to a much lesser extent than IL-12 (Table 1). The lower frequency of naïve CB CD4$^+$ T cells that differentiate into IL-21-expressing cells (approximately 6%) compared with naïve cells from PB (approximately 18%) and tonsil (approximately 12%) possibly reflects the relative immaturity of CB lymphocytes relative to adult lymphocytes.[26–29]

A large proportion of the IL-21$^+$ cells generated from naïve CB or PB CD4$^+$ T cells in the Th1 cultures co-expressed IFN-γ (Figure 4b, Table 1). However, unlike IL-12, acquisition of IL-21 expression after culture with IL-6, IL-21, IL-23 or Th17 conditions was not accompanied by the generation of IFN-γ$^+$ cells (Figures 4b and e). To demonstrate that IL-12-induced differentiation to IL-21$^+$ cells was a direct effect of IL-12 and not an indirect effect of IFN-γ, naïve CB and PB CD4$^+$ T cells were also cultured with IFN-γ. Although IFN-γ increased the frequency of IFN-γ$^+$ cells (Figure 4e), it failed to induce IL-21 expression in CB CD4$^+$ T cells (Figures 4c and d; Table 1). Furthermore, culturing naïve CB CD4$^+$ T cells with IL-12 in the presence of neutralizing anti-IFN-γ mAb had no effect on the induction of IL-21 (%IL-21$^+$ cells with IL-12: 12.2%; %IL-21$^+$ cells with IL-12 plus anti-IFN-γ mAb: 10.8%). These results demonstrate that induction of IL-21 by IL-12 is independent of IFN-γ.

As IL-21 is capable of inducing its own expression, it was possible that IL-12-induced production of IL-21 was mediated by endogenous IL-21. To investigate this, IL-21 induction by IL-12 was determined in the presence of a neutralizing IL-21 Ab. Neutralizing IL-21 had no effect on IL-21 expression in the 'nil' culture, but as expected it abrogated IL-21 expression induced by exogenous IL-21 (Figure 4f). However, IL-21 blockade had a negligible effect on IL-21 expression induced by IL-12 in the Th1 culture (Figure 4g). Thus, IL-12 induces IL-21 expression independently of IL-21.

IL-12-induced IL-21 expression in naïve CD4$^+$ T cells is independent of T-bet and *RORγt*, but correlates with the expression of *BCL-6*

IL-12 induces IFN-γ production by the action of T-bet.[3] As expected, IL-12 and to a lesser extent IFN-γ induced *TBX21* in cultured CB CD4$^+$ T cells (Figure 4g). As the IL-21$^+$ population of activated CB CD4$^+$ T cells was comprised of both IFN-γ$^+$ and IFN-γ$^-$ subsets (Figure 4b), we next questioned whether T-bet was likely to be involved in the ability of IL-12 to induce IL-21$^+$ cells. This was achieved by assessing intracellular expression of T-bet in activated CD4$^+$ T cells partitioned into subsets according to the differential expression of IL-21 and IFN-γ. Although IL-21$^-$IFN-γ$^+$ and IL-21$^+$IFN-γ$^+$ T cells expressed high levels of T-bet, its expression in IL-21$^+$IFN-γ$^-$ T cells was much lower—in fact, it was comparable to IL-21$^-$IFN-γ$^-$ cells (Figure 4h). As *BCL-6* is highly expressed by T_{FH} cells (Figure 2a[7–10,20]), and required for the generation of murine T_{FH} cells,[21,22] we established the effect of IL-21-inducing cytokines on *BCL-6* expression in activated CB CD4$^+$ T cells. Consistent with the acquisition of IL-21, expression of *BCL-6* was increased approximately twofold in the presence of IL-12 or the Th17 culture, to a weaker extent by IL-6, IL-21 and IL-23, but not by culture under Th2 conditions or with IFN-γ (Figure 4i). Thus, these results suggest that the IL-12-mediated generation of IL-21$^+$IFN-γ$^-$ T cells is independent of T-bet, and is probably dependent on another transcription factor such as *BCL-6*.

Several reports have suggested that IL-21 production is a key feature of Th17 cells and that these cells produce the greatest quantities of IL-21.[30] However, in our cultures, it is highly unlikely that the IL-21-expressing cells induced by IL-6, IL-12 or IL-21 resulted from the generation of Th17 cells, because these cytokines failed to induce expressions of IL-17A, IL-22 (data not shown) and *RORC* (Figure 4j) in CB CD4$^+$ T cells. On the other hand, IL-23 or Th17 conditions did induce *RORC*, in addition to IL-21, raising the possibility that these IL-21$^+$ Th cells may belong to the Th17 lineage. This finding also demonstrates that it is IL-23, rather than IL-6, in the Th17 culture that is responsible for inducing *RORC*. Overall, IL-12 appears to be the predominant cytokine that induces T_{FH}-like features in naïve CD4$^+$ T cells, whereas other cytokines can prime these cells for additional fates such as the Th17 lineage.

IL-21$^+$ cells generated *in vitro* by IL-12 exhibit phenotypic and functional features characteristic of T_{FH} cells

When naïve CB CD4$^+$ T cells were cultured *in vitro* with anti-CD3 and anti-CD28 mAb, they upregulated several surface molecules, CXCR5 and ICOS, that are highly expressed on T_{FH} cells (Figures 5a and b; left panels). Expression of these molecules was greatest at 48 h, after which time expression plateaued and then decreased (Figures 5a and b; left panels), indicating the transient nature of these phenotypic changes. In the presence of IL-12 naïve CD4+ T cells from both CB and PB expressed higher levels of CXCR5 (Figure 5a) and ICOS (Figure 5b) than when cultured under non-polarizing conditions. This property was unique to IL-12/Th1 cultures, because exposure of naïve CD4+ T cells to IL-4 (that is, Th2), Th17, IL-6, IL-21 or IL-23 failed to consistently increase the expression CXCR5 or ICOS (Figures 5a and b). Thus, in addition to IL-21 expression, naïve CD4$^+$ T cells cultured

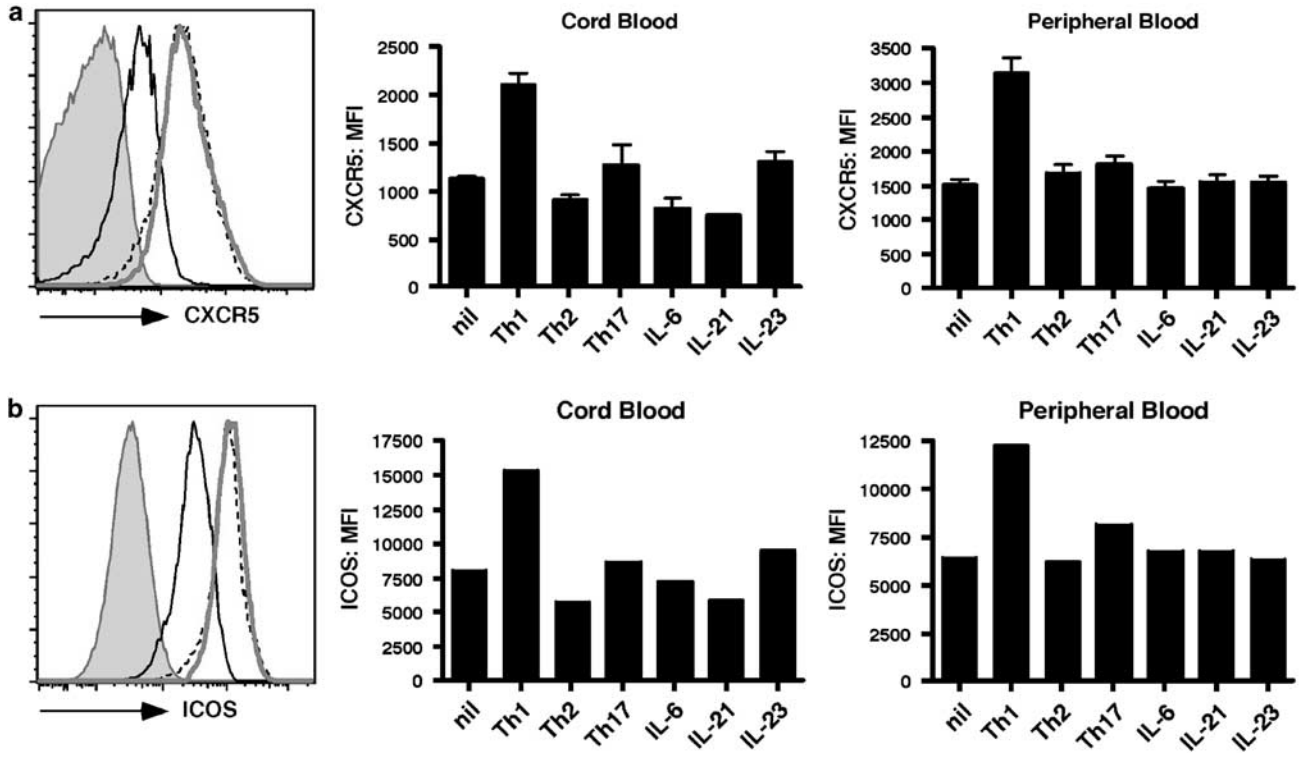

Figure 5 IL-12 induces increased expression of CXCR5 and ICOS on naïve CD4+ T cells. Left panel: Expression of CXCR5 (**a**) and ICOS (**b**) on cord blood (CB) CD4+ T cells was determined *ex vivo* (shaded histogram) and after 1 (open histogram), 2 (dashed histogram) and 3 (grey outline histogram) days in culture under Th1 conditions. Middle and right panels: $CD4^+CD45RA^+$ cord blood (middle) and peripheral blood (right) T cells were stimulated with neutral (nil) conditions or in the presence of IL-12 (Th1), IL-4 (Th2), IL-1β–IL-6–IL-23 (Th17), or IL-6, IL-21 or IL-23 alone for 3–4 days. After this time the MFI of CXCR5 and ICOS expression were determined.

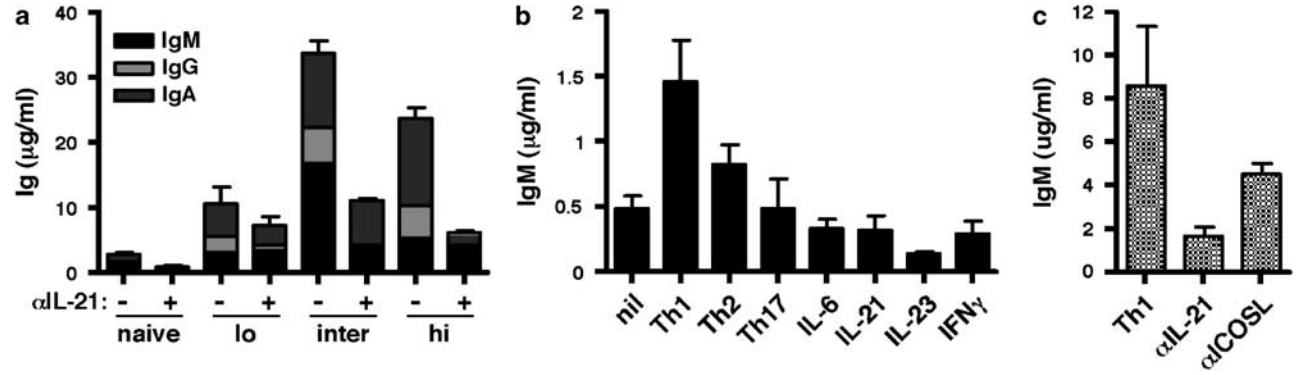

Figure 6 Naïve CD4+ T cells primed with IL-12 provide improved help for T-cell dependent differentiation of B cells into Ig-secreting cells. (**a**) Sort-purified tonsillar CD4+ naïve, $CXCR5^{lo}$, $CXCR5^{inte}$ and $CXCR5^{hi}$ T_{FH} subsets were treated with mitomycin C and then co-cultured with autologous naïve B cells in the presence of anti-CD3 and anti-CD28 mAb without (−) or with (+) a neutralizing polyclonal anti-IL-21 antisera. Secretion of IgM, IgG and IgA was determined after 10 days of culture. (**b**) PB naïve CD4+ T cells were cultured for five days under the indicated conditions. The cells were then harvested, treated with mitomycin C and co-cultured with allogeneic naïve B cells in the presence of TAE beads. Secretion of IgM was determined after seven days of culture. (**c**) PB naïve CD4+ T cells cultured in the presence of IL-12 (Th1) for five days were then treated with mitomycin C and co-cultured with allogeneic naïve B cells in the presence of TAE beads alone or together with neutralizing anti-IL-21 antisera or anti-ICOS-L mAb. Secretion of IgM was determined after eight days of culture.

under Th1 conditions acquired phenotypic features of T_{FH} cells equipping them with a heightened ability to provide help to B cells.

A signature feature of T_{FH} cells is their ability to induce the differentiation of B cells into Ig-secreting cells.[23] This has been established by demonstrating that $CD4^+CXCR5^+$ T cells from human tonsils are superior at providing help to co-cultured B cells for Ig secretion than $CD4^+CXCR5^-$ T cells.[4,5] To extend these observations, we tested the relative abilities of naïve, $CXCR5^{lo}$, $CXCR5^{inter}$ and $CXCR5^{hi}$ subsets of tonsillar $CD4^+$ T cells to activate co-cultured B cells. Naïve $CD4^+CD45RA^+CXCR5^-$ T cells induced only low amounts of Ig secretion by co-cultured B cells. In contrast, all three subsets of $CD4^+CD45RA^-$ T cells induced 5–10-fold higher levels of Ig secretion than naïve B cells, with the effect of the $CXCR5^{inter}$ and $CXCR5^{hi}$ subsets exceeding that of the $CXCR5^{lo}$ cells (Figure 6a). When endogenous IL-21 was

neutralized by a blocking polyclonal anti-IL-21 Ab, Ig secretion was reduced, and the extent of inhibition correlated with IL-21 production by CD45RA$^-$ CD4$^+$ T-cell subsets (Figure 6a). Thus, IL-21 production by T_{FH} cells represents a major mechanism, by which they support the differentiation of B cells into Ig-secreting cells.

On the basis of this result, we were interested in determining whether the increased production of IL-21 by naïve CD4$^+$ T cells cultured with IL-12, together with their heightened expression of ICOS, endowed these cells with improved T-helper function. When naïve PB CD4$^+$ T cells were cultured with IL-12, their ability to induce Ig secretion by naïve B cells was approximately threefold greater than that of cells cultured without IL-12, or under the alternative polarizing conditions (Figure 6b). It is important to note that the enhancing effect of IL-12-primed CD4$^+$ T cells on Ig secretion could be dramatically suppressed by blocking endogenous IL-21 or inhibiting ICOS–ICOS ligand interactions (Figure 6c). Thus, not only does IL-12 induce naïve CD4$^+$ T cells to resemble T_{FH}-like cells with respect to cytokine (IL-21) expression and phenotype (CXCR5, ICOS), it also provides these cells with T_{FH} function with respect to the provision of help to B cells for their differentiation into Ig-secreting cells.

DISCUSSION

The objectives of this study were twofold. First, we aimed to provide a detailed characterization of human T_{FH} cells. Whereas initial phenotypic[4] and microarray analysis of human CD4$^+$CXCR5$^+$ T cells revealed important features of T_{FH} cells and identified signature genes,[6,8,9] these studies relied on the analysis of all CXCR5$^+$ T cells[4] or predominantly the CD57$^+$ subset.[6,8,9] Furthermore, the data derived from microarray studies were rarely confirmed by q-PCR or at the protein level. Finally, CXCR5 itself is heterogeneously expressed on human CD4$^+$ T cells, suggesting the existence of CXCR5^{lo}, CXCR5^{inter} and CXCR5^{hi} subsets,[9] and the validity of delineating CXCR5$^+$ T cells into subsets based on CD57 expression has been questioned by several groups.[8,9] For these reasons, it was important to adopt a new approach to characterize human tonsillar CD4$^+$ T cell subsets that made no assumptions about the co-expression of other surface molecules.

To achieve this, we analyzed tonsillar CD4$^+$ T cells according to the differential expression of CD45RA and CXCR5. This identified four populations of CD4$^+$ T cells: naïve CD45RA$^+$CXCR5$^-$, CD45RA$^-$CXCR5^{lo}, CD45RA$^-$CXCR5^{inter} and CD45RA$^-$CXCR5^{hi} cells. Detailed phenotypic, molecular and functional analysis of these subsets provided important insight into the nature of human effector CD4$^+$ T cells. First, CD4$^+$CXCR5^{hi} T cells uniformly expressed the highest levels of ICOS, PD-1, CD40L, OX2 (CD200) and SAP. They also exhibited the greatest expression of *BCL-6* and contained the highest frequency of cells co-expressing IL-4 and IL-21 after *ex vivo* stimulation. Histological analyses of human tonsils have established that CD4$^+$ T cells expressing the most abundant levels of CD40L,[31] PD-1, SAP[32] and ICOS[4,33] localize to GCs. It is interesting to note that PD-1 and SAP were largely co-expressed within GC CD4$^+$ T cells and approximately 20% of these were also BCL-6$^+$.[32] Taken with our phenotypic analysis, this suggests that CD4$^+$CXCR5^{hi} T cells would be positioned within GCs, and thus correspond to bona fide T_{FH} cells. It is important to emphasize that although previous studies have indicated that Th17 cells produce the greatest amounts of IL-21,[30] our results exclude any possibility that CXCR5^{hi} CD4$^+$ T cells are Th17 cells because they lacked expression of the signature Th17 molecules *RORC*, IL-17, IL-22 and CCR6.

The elevated expressions of ICOS, CD40L and IL-21 by CD4$^+$CXCR5^{hi} T cells is likely to contribute directly to their ability to provide help to B cells.[9,34–36] Similarly, our finding that CD4$^+$CXCR5^{hi} T cells expressed the highest amounts of BTLA, CD95, CXCR4 and CD126 (IL-6R) would predict important roles for these molecules in T_{FH} cell biology. Heightened expression of inhibitory (BTLA and CD200) and death (CD95 and PD-1) receptors by T_{FH} cells is likely to be a mechanism to control their expansion and/or function, as evidenced by the fact that aberrant regulation of T_{FH}-cell differentiation can cause T-cell lymphoma in humans[37] and lupus-like disease in mice.[20] Consistent with this, T_{FH} cells have been found to accumulate in the blood of patients with the autoimmune conditions systemic lupus erythematosus and Sjogren's syndrome.[38] The expression of CXCR4 is more highly expressed on GC B cells than on follicular B cells. This increased expression of CXCR4, together with constitutive CXCR5 expression, on GC B cells is functionally significant because CXCR4-deficiency affected the positioning of B cells within a GC.[39] Based on this, we postulate that co-expression of CXCR4 and CXCR5 by T_{FH} cells provides a mechanism for their localization to GC, thereby facilitating their interaction with GC B cells. The sustained expression of CD126 would be consistent with IL-6 having a role in supporting the maturation or function of T_{FH} cells, as recently reported for mice[10,12,13,25] and demonstrated here for humans. In contrast, the lack of expression of CD127 (IL-7R), CCR6, CCR7 and CXCR3 predicts that CD4$^+$CXCR5^{hi} T cells would be insensitive to the effects of ligands for these cytokine and chemokine receptors. Indeed it has been previously reported that effector CD4$^+$ T cells that localize to the B-cell follicle and have unique B-helper functions could be identified by the loss of CD127.[35]

The prominent expression of SAP by CD4$^+$CXCR5^{hi} T cells is consistent with defects in T_{FH} cells in humans with mutations in *SH2D1A*, which encodes SAP, and *sap*$^{-/-}$ mice.[19,40–42] Of the four CD4$^+$ T cell subsets examined, the CD4$^+$CXCR5^{hi} cells expressed the highest level of the SAP-binding receptor CD84. Although the expression of Ly9 and NTB-A on CD4$^+$CXCR5^{hi} T cells exceeded that of naïve cells, it was comparable to that observed for CXCR5^{lo} and CXCR5^{inter} subsets. Thus, it is unclear which SAP-binding receptor is most critical for humoral immune responses. The elevated expression of CD84 on human CD4$^+$CXCR5^{hi} T cells makes this receptor a likely candidate. It is also possible, however, that SAP deficiency impairs T_{FH}-cell function and subsequent B-cell responses by compromising signals delivered to CD4$^+$ T cells through several SAP-binding receptors.[19] This awaits the generation and analysis of mice rendered deficient in CD84 singly and in combination with other candidate receptors.

Although CD4$^+$CXCR5^{hi} T cells were reasonably uniform with respect to surface phenotype and the predominant expression of a single T-cell lineage transcription factor, the CXCR5^{lo} and CXCR5^{inter} subsets were notably heterogeneous. The CD45RA$^-$CXCR5^{lo} subset expressed the greatest levels of *TXB21, GATA3, RORC* and *FOXP3*, and contained the greatest frequencies of cells expressing or producing IFN-γ, IL-13 and IL-17, and CCR6 and CXCR3. It is interesting to note that CD4$^+$CXCR5^{lo} T cells also contained a substantial fraction of IL-4 and IL-21-expressing cells, but unlike CD4$^+$CXCR5^{hi} T-cells expression of these cytokines by CD4$^+$CXCR5^{lo} cells segregated into independent populations. It is likely that the IL-4- and IL-13-producing cells within the CXCR5^{lo} population represent canonical Th2 cells, whereas the IL-4$^+$ cells in the CXCR5^{hi} population are true T_{FH} cells. This analysis also suggests that IL-13 is not produced by T_{FH} cells. Furthermore, $>$50% of IL-17$^+$ CD4$^+$CXCR5^{lo} cells co-expressed IL-21. Collectively, this demonstrates that the CXCR5^{lo} population of tonsillar CD4$^+$ T cells is comprised of Th1 (IFN-γ$^+$*TBX21*$^+$), Th2 (IL-4$^+$IL-13$^+$IL-21$^-$*GATA3*$^+$), Th17 (IL-17$^+$IL-21$^\pm$IL-22$^+$*RORC*$^+$) and

Treg (*FOXP3*$^+$) cells. Expression of these cytokines, transcription factors and chemokine receptors was also noted for some CD4$^+$CXCR5^{inter} T cells, however to a lesser extent than the CD4$^+$CXCR5^{lo} subset. Consequently, effector Th1, Th2, Th17 and Treg cells can also upregulate the expression of CXCR5. This may explain previous observations that some CD4$^+$CXCR5$^+$ T cells are suppressive[43] or produce substantial quantities of IFN-γ,[6] an observation not made for the CXCR5^{hi} T_{FH} cells identified here. Based on this, it will be interesting to determine whether the B-cell zone homing 'Th17' cells reported to underlie disease development in a murine model of lupus[44] are actually Th17 cells or rather correspond to the CXCR5^{inter} subset of CD4$^+$ T cells that not only retain the capacity to produce inflammatory cytokines—IFN-γ and IL-17—but also have the ability to migrate into GCs due to low-level expression of CXCR5.

PRDM1/Blimp-1 is well-characterized for its necessary role in generating plasma cells.[24] Recently, *PRDM1* was found to inhibit the generation of murine T_{FH} cells from naïve precursors by antagonizing the action of Bcl-6.[22] The reciprocal expression of *PRDM1* and *BCL-6* in human CD4$^+$ CXCR5^{lo} and CXCR5^{hi} T cells, respectively, resembles that of plasma cells (Blimp-1$^+$Bcl-6$^-$) and GC B cells (that is, Bcl-6$^+$Blimp-1$^-$). These transcriptional repressors, therefore, probably have a parallel function in regulating the commitment of naïve human CD4$^+$ T cells into Blimp-1$^+$Bcl-6$^-$ cells capable of becoming Th1, Th17 or Treg effector CD4$^+$ T cells or into Bcl-6$^+$Blimp-1$^-$ T_{FH} cells.

The second objective of our investigation was to identify requirements necessary for the differentiation of human naïve CD4$^+$ T cells into T_{FH}-like cells *in vitro*. This resulted in the discovery that several cytokines—IL-12, IL-6, IL-23 and IL-21 itself—could induce IL-21 production by naïve CD4$^+$ T cells in human tonsils, as well as CB and PB. The ability of IL-12 to induce IL-21 expression in human naïve CD4$^+$ T cells is striking in as much that IL-12 had no effect on IL-21 production by murine CD4$^+$ T cells,[10,12,25] thus highlighting the species-specific effect of IL-12. Although human and murine CD4$^+$ T cells differed with respect to the ability of IL-12 to induce IL-21 production, our findings confirmed previous observations that IL-6 and IL-21 were also capable of priming naïve murine CD4$^+$ T cells to differentiate into IL-21-expressing cells.[10–12,25] The mechanism by which IL-12 induces IL-21 expression in human CD4$^+$ T cells is unknown. As IL-12 also induced *BCL-6*, and IL-21$^+$IFN-γ$^-$ cells lacked T-bet, it is tempting to speculate that this process involves Bcl-6 and not T-bet. Our findings largely confirm those recently reported by Schmitt *et al*,[45] who also reported that IL-12 was the predominant cytokine responsible for inducing IL-21 production by human naïve CD4$^+$ T cells, whereas IL-21 and IL-23 also shared this property albeit it to a much lesser extent. However, our studies substantially extended these findings by correlating induction of IL-21 expression with increased *BCL-6* and CXCR5 expression. This ability of IL-12 to induce increased expression of CXCR5 may be important in localising these IL-21$^+$ CD4$^+$ T cells to the B-cell follicle, where they are positioned to provide B cell help.[23,42,46] Moreover, our data highlight the importance of monitoring expression of other transcription factors, as it is possible that the CD4$^+$ T cells induced to express IL-21 by IL-23 may actually belong to the Th17 lineage rather than the T_{FH} lineage, as IL-23 is known for its ability to induce both *IL-21* and *RORC*—that is, features of Th17 cells—in naïve human CD4$^+$ T cells.[46] Further analysis of RORγt expression by IL-21$^+$ cells induced by different *in vitro* culture conditions will help resolve this issue. In contrast to our results, Schmitt *et al*[45] reported that IL-6 had no effect on IL-21 induction by human naïve CD4+ T cells. It is unclear why this difference was observed, but may reflect the *in vitro* culture systems used. It will therefore be important to address the effects of IL-6 on the formation of human T_{FH} cells.

The physiological source of IL-12 for the differentiation of naïve CD4$^+$ T cells into IL-21-expressing cells is likely to be activated DC,[45] which are well-characterized for their ability to produce high quantities of IL-12 after stimulation through CD40 or TLRs.[47] Although our finding that IL-12 is the predominant cytokine responsible for inducing IL-21 in human naïve CD4$^+$ T cells may appear surprising, and may be difficult to reconcile with its central role in inducing Th1-mediated immune responses, it is consistent with the critical role of DCs in both directing the differentiation of naïve T cells into specific effector fates and in regulating humoral immune responses.[46,47] In light of this, it is of interest that DCs from patients with common variable immunodeficiency are impaired in IL-12 production.[48] It is therefore possible that this deficit in IL-12 production *in vivo* would contribute to the defect in humoral immune responses that are characteristic of common variable immunodeficiency. Our proposal, together with that of Schmitt *et al*,[45,46] that IL-12 is involved in generating T_{FH} cells is supported by earlier findings that IL-12 can augment expression of CD40L[49,50] and ICOS[51]—additional features of T_{FH} cells (Figure 1[4,5,9,20,35])—on *in vitro* activated CD4$^+$ T cells, and that Ab production by B cells co-cultured with IL-12-primed CD4$^+$ T cells was in part dependent on IL-12 sustaining expression of CD40L.[50]

A model for the generation of T_{FH} cells involves an initial interaction between Ag-specific CD4$^+$ naïve T cells and DCs,[47] which induces the expression of IL-21 via production of IL-12 (humans only) and/or IL-6 (humans and mice: Figure 4[10,12,13,25]). These IL-21$^+$CD4$^+$ T cells then interact with Ag-specific B cells, which dictate their further maturation towards a T_{FH}-cell fate in an ICOS–ICOS-L dependent manner.[10,11,42,45,46] From this, we would propose that IL-12-induced IL-21$^+$ human CD4$^+$ T cells correspond to precursor T_{FH} cells that, after CXCR5-mediated migration to the B-cell zone and ICOS-mediated interactions with cognate B cells, will undergo further differentiation and acquire key features of T_{FH} cells. The CD4$^+$ T cells generated *in vitro* by IL-12 resembled tonsillar CXCR5$^{lo/inter}$CD4$^+$ T cells in as much that they expressed IFN-γ in addition to IL-21, had relatively low levels of ICOS and CXCR5, and lacked IL-4. It is likely that after exposure to the appropriate microenvironment, these 'Th1-like' cells will acquire the capacity of becoming a bona fide T_{FH} cell. This is supported by the observation that human tonsillar CD4$^+$CXCR5$^{lo/inter}$ T cells produce substantial amounts of IFN-γ when cultured *in vitro*, but alter their cytokine profile after co-culture with autologous GC B cells such that they acquired the ability to produce IL-4 while concomitantly downregulating IFN-γ production.[16] This suggests a degree of plasticity within the differentiation program of CD4$^+$ T cells such that one population of effector cells (for example, Th1 cells) can acquire feature of another subset (for example, T_{FH} cells). This is consistent with recent murine studies showing that T_{FH} cells could differentiate from Th2 or Foxp3$^+$ CD4$^+$ T cells in lymph nodes or Peyer's patches.[52–55] Retaining this level of plasticity would enable the rapid development of T_{FH} cells when required and would be less exhausting than differentiation from a naïve precursor. Alternatively, sustained production of cytokines other than IL-21 by pre-T_{FH} cells may regulate or shape the immune response. Thus, although IL-21 would be sufficient to initiate B-cell activation and differentiation, other cytokines such as IFN-γ and IL-4 could fine-tune the immune response. Indeed, IL-4- and IFN-γ-expressing murine T_{FH} cells could induce isotype switching to IgG1 and IgG2a, respectively, *in vivo*.[52] Similarly, we have shown that IL-21 can induce human B cells to undergo Ig isotype switching to IgG3 and

IgA, but IL-4 skews the IL-21-induced response towards IgG1 and away from IgA.[36] Thus, production of IL-21 in association with other cytokines may be required to modulate Ab production by B cells.

Key unanswered questions in the field of T_{FH} cells include identifying mechanisms that guide the migration of precursor T_{FH} cells into B-cell follicles, and the relationship between T_{FH} cells and other effector cell subsets—Th17, Treg, Th1 and Th2 cells—which share similar features with and/or can differentiate into T_{FH} cells.[46] Our detailed characterization of distinct effector $CD4^+$ T-cell subsets generated *in vivo* and the identification of factors involved in the early commitment of naïve $CD4^+$ T cells to a T_{FH}-like fate represent important advances in our understanding of the biology of human $CD4^+$ T-cell differentiation. Given the role of T_{FH} cells in Ab-mediated immune response, this information can be used to address these outstanding questions with the ultimate objective being the ability to modulate these cells for improving the development of vaccines or novel therapies for treating conditions, such as immunodeficiencies, autoimmunity and malignancy.

METHODS

Human samples

Human tonsils were obtained from routine tonsillectomy at the St Vincent's Hospital (Darlinghurst, NSW, Australia). CB samples were obtained from Nepean Hospital (Nepean, NSW, Australia). PB buffy coats were obtained from the Australian Red Cross Blood Service. All studies were approved by institutional Human Research Ethics Committees.

Antibodies

The following Abs were used: Alexa-647-conjugated anti-IL-21 mAb, biotinylated anti-BTLA and anti-ICOS mAb, PE-conjugated anti-OX40, anti-CD4 and anti-T-bet mAb, Pacific blue-conjugated anti-IL-10 mAb, PE-Cy7-conjugated anti-IL-4 mAb, PerCP-Cy5.5-anti-IFNγ, FITC-conjugated anti-IL-17A and anti-CD45RA mAb and neutralizing anti-ICOS-L mAb (eBioscience, San Diego, CA, USA); Alexa 647-anti-CXCR5 mAb, APC-anti-CXCR3 mAb, PE-anti-CCR6, anti-CD69, anti-CD95, anti-CD126, anti-CD154, anti-CXCR4, anti-OX2 and anti-IL-2 mAb, FITC-anti-CD57 mAb and neutralizing anti-IFN-γ mAb (Becton Dickinson, San Jose, CA, USA); biotinylated anti-PD-1, PE-anti-CCR7 mAb (R&D systems, Minneapolis, MN, USA); PE-anti-CD127 mAb (Beckman Coulter, Miami, FL, USA); anti-SAP mAb (Abnova, Taipei City, Taiwan) was conjugated to Alexa-647 (Invitrogen, Carlsbad, CA, USA); neutralizing anti-IL-21 Ab (Peprotech, Rocky Hill, NJ, USA).

Isolation of $CD4^+$ T cells

$CD4^+$ T cells were isolated from human tonsils, CB and PB using Dynal beads as per manufacturer's instructions. Tonsillar $CD4^+$ T cells were labelled with anti-CD45RA and anti-CXCR5 mAbs and then sorted into populations of $CD45RA^+CXCR5^-$, $CD45RA^-CXCR5^{lo}$, $CD45RA^-CXCR5^{inter}$ and $CD45RA^-CXCR5^{hi}$ cells using a FACS ARIA (Becton Dickinson). Naïve CD4+ T cells were isolated from PB by sorting $CD45RA^+CCR7^+CXCR5^-$ cells. Purified CB $CD4^+$ T cells were labelled with CD45RA microbeads and $CD45RA^+$ T cells were isolated using an AutoMACs (Miltenyi Biotech, Bergisch Gladbach, Germany). Purity of the recovered $CD4^+$ T-cell subsets was >97%.

Ex vivo analysis of phenotype and expression of cytokines and transcription factors by tonsil $CD4^+$ T-cell subsets

Phenotype: Tonsillar $CD4^+$ T cells were labelled with anti-CD45RA and anti-CXCR5 mAbs, and either an isotype control or a mAb to the indicated cell surface molecule. Populations corresponding to $CD45RA^+CXCR5^-$, $CD45RA^-CXCR5^{lo}$, $CD45RA^-CXCR5^{inter}$ and $CD45RA^-CXCR5^{hi}$ cells were identified by electronic gating, and expression of the molecules of interest was determined. The expression of SAP was determined after surface staining with anti-CD45RA and anti-CXCR5 mAbs and then fixation and permeabilization with formaldehyde and saponin. CD40L expression was determined after culturing sort-purified subsets of tonsil $CD4^+$ T cells with PMA (100 ng ml^{-1}) and ionomycin (750 ng ml^{-1}) for 2 h.

Cytokine expression: Sorted tonsil $CD4^+$ T-cell subsets were stimulated *in vitro* with PMA (100 ng ml^{-1}) and ionomycin (750 ng ml^{-1}) for 6 h, with brefeldin A (10 μg ml^{-1}) being added after 2 h. The cells were then fixed with formaldehyde and expression of cytokines detected by intracellular staining using saponin to permeablize the cells and cytokine-specific mAbs, as previously described.[40,56]

Transcription factors: Total RNA was extracted from sorted tonsil $CD4^+$ T-cell subsets immediately after isolation using the RNeasy kit as per manufacturer's instructions (Qiagen, Doncaster, VIC, Australia), and transcribed into cDNA using random hexamers and Superscript III (Invitrogen). All real-time PCR primers (Integrated DNA Technologies, Coralville, IN, USA) were designed using the Roche UPL Primer Design Program. The primer sequences, Roche UPL probes and size of each amplicon are as follows: *IL-21*—fwd: AGGAAA CCACCTTCCACAAA; rev: GAATCACATGAAGGGCATGTT; UPL probe: 7; amplicon: 68; *BCL-6*—fwd: GAGCTCTGTTGATTCTTAGAACTGG; rev: GCC TTGCTTCACAGTCCAA; UPL probe: 9; amplicon: 110; *PRDM1* (Blimp-1)—fwd: ACGTGTGGGTACGACCTTG; rev: CTGCCAATCCCTGAAACCT; UPL probe: 67; amplicon: 68; *TBX21* (T-bet)—fwd: TGTGGTCCAAGTTTAATC AGCA; rev: TGACAGGAATGGGAACATCC; UPL probe: 9; amplicon: 77; *GATA3*—fwd: GCTTCGGATGCAAGTCCA; rev: GCCCCACAGTTCACAC ACT; UPL probe: 8; amplicon: 70; *RORC*—fwd: CAGCGCTCCAACATCTTCT; rev: CCACATCTCCCACATGGACT; UPL probe: 69; amplicon: 69; *FOXP3*—fwd: ACCTACGCCACGCTCATC; rev: TCATTGAGTGTCCGCTGCT; UPL probe: 50; amplicon: 65; *GAPDH*—fwd: CTCTGCTCCTCCTGTTCGAC; rev: ACGACCAAATCCGTTGACTC; UPL probe: 60; amplicon: 112. Real-time PCR was carried out using the Roche LightCycler 480 Probe Master Mix and the Roche Lightcycler 480 System with the following conditions: denaturation at 95 °C for 10 mins, amplification for 45 cycles at 95 °C for 10 secs, 65 °C for 30 secs and 72 °C for 5 secs, and cooling at 40 °C for 30 secs. All reactions were standardized to the level of expression of the house-keeping gene *GAPDH*.

Cell cultures

Tonsil, CB and PB $CD4^+$ T cell subsets were cultured with T-cell activation and expansion (TAE) beads (Miltenyi Biotech) and recombinant human (rh) IL-2 (20 U ml^{-1}, Millipore, North Ryde, NSW, Australia) alone (nil culture) or under the following polarizing conditions: Th1: IL-12 (20 ng ml^{-1}, R&D systems); Th2: IL-4 (100 U ml^{-1}, gift from Dr Rene de Waal Malefyt, DNAX Research, Palo Alto, CA, USA); Th17: IL-1β (20 ng ml^{-1}, Peprotech)[40,56–58] IL-6 (50 ng ml^{-1}, Peprotech) and IL-23 (20 ng ml^{-1}, eBioscience); or with IL-6 (50 ng ml^{-1}), IL-21 (50 ng ml^{-1}, Peprotech), IL-23 (20 ng ml^{-1}) or IFN-γ (1000 U ml^{-1}, Becton Dickinson) alone for five days. In some cultures a neutralizing IL-21 or anti-IFN-γ Ab (15 μg ml^{-1}) was added at day 0. After this time, the expression of intracellular cytokines and transcription factors and surface phenotype of the cells was determined as detailed above. Cytokine secretion was determined using cytokine specific ELISA, as previously described.[56]

T–B cell co-cultures

Tonsil $CD4^+$ T cells were sorted into subsets of $CD45RA^+CXCR5^-$, $CD45RA^-CXCR5^{lo}$, $CD45RA^-CXCR5^{inter}$ and $CD45RA^-CXCR5^{hi}$ cells, treated with mitomycin C and then co-cultured with autologous naïve B cells in the presence of TAE beads.[34,40] Endogenous IL-21 was neutralized by the inclusion of anti-IL-21 polyclonal Ab (15 μg ml^{-1}). Naïve $CD4^+$ T cells were isolated from PB by sorting $CD45RA^+CCR7^+$ cells and then cultured under the conditions outlined above (see Cell cultures section). After five days, the cells were harvested, treated with mitomycin C and then co-cultured with allogeneic tonsil naïve B cells[40] in the presence of TAE beads. In some experiments, the effect of inhibiting IL-21 or ICOS–ICOS-L interactions were examined by adding neutralizing anti-IL-21 polyclonal Ab or anti-ICOS-L mAb. Secretion of Ig was determined after 7–10 days by Ig heavy-chain specific ELISA.[34,40]

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