IL-6 Triggers IL-21 production by human CD4\(^+\) T cells to drive STAT3-dependent plasma cell differentiation in B cells

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Interleukin (IL)-21-producing CD4\(^+\) T cells are central to humoral immunity. Deciphering the signals that induce IL-21 production in CD4\(^+\) T cells and those triggered by IL-21 in B cells are, therefore, of importance for understanding the generation of antibody (Ab) responses. Here, we show that IL-6 increased IL-21 production by human CD4\(^+\) T cells, particularly in those that express the transcriptional regulator B cell lymphoma (BCL)6, which is required in mice for the development of C-X-C chemokine receptor type 5 (CXCR5\(^+\)) IL-21-producing T follicular helper (T\(_{FH}\)) cells. However, retroviral overexpression of BCL6 in total human CD4\(^+\) T cells only transiently increased CXCR5, the canonical T\(_{FH}\)-defining surface marker. We show here that IL-21 was required for the induction of Ab production by IL-6. In IL-21-treated B cells, signal transducer and activator of transcription (STAT)3 was required for optimal immunoglobulin production and upregulation of PR domain containing 1 (PRDM1\(^+\)), the master plasma cell factor. These results, therefore, demonstrate the critical importance of STAT3 activation in B cells during IL-21-driven humoral immunity and suggest that BCL6 expression, although not sufficient, may serve as a platform for the acquisition of a T\(_{FH}\)-like phenotype by human CD4\(^+\) T cells.

Immunology and Cell Biology (2012) 90, 802–811; doi:10.1038/icb.2012.17; published online 10 April 2012

Keywords: human; IL-6; IL-21; STAT3; T\(_{FH}\) cells; BCL6

During an adaptive immune response, activated CD4\(^+\) T helper cells provide help to B cells by cell surface signals and by secreting cytokines to promote B cell activation, induction of immunoglobulin (Ig) isotype switching and terminal differentiation into Ig-producing plasma cells. There is a considerable diversity in T helper subsets that provide B cell help. Cytokines present during initial CD4\(^+\) T cell activation enact distinct transcriptional programs that control differentiation of T helper subsets.\(^1\) In particular, T follicular helper cells (T\(_{FH}\)) have also been proposed as a distinct, dedicated B cell helper T helper lineage.\(^2\) T\(_{FH}\) cells are defined by their presence in the B cell areas of germinal centers, high sustained expression of the GC-homing chemokine receptor CXCR5 and ability to promote differentiation of B cells to plasma cells.\(^3,4\)

Interleukin (IL)-21 is a key cytokine produced by T\(_{FH}\) cells.\(^5,6\) IL-21 is a common-\(\gamma\) chain cytokine with a critical function in promoting recombination and somatic hypermutation.\(^15\) In contrast, BLIMP1 suppresses proliferation and enhances the protein production machinery of the endoplasmic reticulum necessary for high-level Ig secretion by plasma cells.\(^13,16\)

The BCL6–BLIMP1 axis also controls the development of T\(_{FH}\) cells.\(^2\) BCL6 is required for T\(_{FH}\) cell differentiation\(^17–19\) and Blimp-1 counteracts this process.\(^17\) Accordingly, human T\(_{FH}\) cells also express high levels of BCL6 and low levels of PRDM1.\(^20,21\) Although it is not completely clear how BCL6 and BLIMP1 levels are controlled during T\(_{FH}\) differentiation, there is evidence for cytokine and cellular help from antigen-presenting cells (APCs) in this process.\(^21,22\)

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Received 5 August 2011; revised 28 February 2012; accepted 29 February 2012; published online 10 April 2012
IL-6 has been recently shown to activate IL-21 production by murine CD4\(^+\) T cells.\(^{23-25}\) Given that similar transcriptional mechanisms and cytokines (that is, IL-6, IL-21, STAT3, BLIMP1 and BCL6) are involved in both T and B cells in the development of the antibody (Ab) response, we assessed cell-specific responses, and requirements for these factors during induction of Ig production and T\(\text{FH}\) development. First, we describe a role for IL-6 in promoting Ab production in complex cellular conditions through the enhanced production of IL-21 by IL-6-exposed CD4\(^+\) T cells. Second, we have determined a requirement for STAT3 expression in IL-21-mediated B cell cell differentiation at the level of Ig production and transcriptional activation of PRDM1 in normal human B cells. Lastly, in line with the ability of IL-6 to promote changes in CD4\(^+\) T cells consistent with T\(\text{FH}\) cells, (that is, enhanced IL-21 production and expression of BCL6), we also observed that overexpression of BCL6 itself increased expression of the T\(\text{FH}\)-associated markers, CXCR5 and CXCR4. Thus, IL-21 and STAT3 are required for plasma cell differentiation and Ab production by CD4\(^+\) T cells. Our results also reveal that BCL6 expression is involved in the early acquisition of the human CXCR5\(^+\) T\(\text{FH}\) phenotype.

RESULTS

The cytokine environment during T cell-dependent activation of B cells can strongly influence Ab production. We, therefore, directly compared the B cell helper activity of T cell- with non-T cell-derived cytokines in response to T cell activation in a multicellular context. We found that in contrast to IL-2 or IL-4, both IL-6 and IL-21 significantly enhanced Ig secretion in phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) cultures (Figure 1). B and T cell frequencies in the cultures (9±2% and 70±10%, respectively) were not affected by any cytokine during the culture (not shown). Similar results were obtained with anti-CD3/anti-CD28 stimulation (not shown). These results suggested that IL-6 or IL-21 were able to elicit Ig production by B cells in the presence of activated T cells.

To determine B cell-specific effects of these cytokines, we cultured B cells with these cytokines and irradiated CD40L-expressing L cells (CD40L-L cells) to simulate T cell help in the GC. IL-21-induced robust plasma cell differentiation, as evidenced by the appearance of CD38\(^+\)CD20\(^{lo}\) cells (Figure 2a, upper panels). Likewise, IL-21-treated B cells exhibited a CD138\(^+\)CD19\(^{lo}\) phenotype (Figure 2a, lower panels). Neither IL-2 nor IL-4 increased plasma cell formation in CD40L-activated B cells. IL-6 has been described to induce plasmablast survival, but despite IL-6R\(\alpha\) expression on CD40L-activated B cells, IL-6 did not induce plasma cell differentiation (Figure 2a). We examined cell number in these cultures and found that although IL-4 did not induce differentiation, this cytokine modestly increased total cell number (Figure 2b). IL-21 promoted robust B cell proliferation, whereas IL-6 did not (Figure 2b). We then analyzed Ig production and, found that only IL-21 significantly increased Ig production on a per cell basis, whereas neither IL-4 nor IL-6 had this effect (Figure 2c). To further demonstrate that IL-21 specifically mediated plasma cell differentiation, we also examined the gene expression level of PRDM1, the gene encoding BLIMP1.\(^{26}\) IL-21 strongly upregulated PRDM1 expression in CD40L-activated human B cells (Figure 2d). IL-4 and IL-6 did not promote PRDM1 expression, and IL-2 had only a minor effect though not statistically significant. These results are consistent with the known role for IL-21 in the initiation of human plasma cell differentiation,\(^{10,27}\) but do not support a role for IL-6 acting directly on B cells to initiate plasma cell differentiation and promote Ig production.

As the addition of IL-6 did not directly trigger Ig production by activated purified B cells, but did so in a multicellular context when activated T cells were present, we hypothesized that the effect of IL-6 was indirect, presumably mediated by T cells. In the GC, activated T\(\text{FH}\) cells produce large quantities of IL-21,\(^{5,6}\) the most potent known initiator of human plasma cell differentiation.\(^{28,29}\) We, therefore, investigated whether IL-6 could induce the production of IL-21 by human CD4\(^+\) T cells. Total CD4\(^+\) T cells purified from peripheral blood of healthy donors were activated with PHA in the presence or absence of IL-6. Exogenous IL-6 as well as IL-21 induced IL-21 gene expression (Figure 3a). In addition, increased levels of secreted IL-21 protein were detected in supernatants of CD4\(^+\) T cells activated in the presence of IL-6 (Figure 3b).

The GC-specific transcriptional repressor BCL6 is highly expressed in IL-21-secreting human tonsillar T\(\text{FH}\) cells,\(^{20,21}\) and has a critical function in T\(\text{FH}\) cell programming in mice.\(^{5,17,18}\) As IL-6 and IL-21 increased IL-21 mRNA and protein in total CD4\(^+\) T cells, we analyzed whether IL-6 could influence BCL6 levels. In accordance with its positive effect on IL-21 production, IL-6 significantly (2.9±0.6-fold) increased BCL6 transcript levels, whereas IL-21 did not affect BCL6 levels (Figure 3c). Our results suggest that IL-6 promotes IL-21 production and upregulation of BCL6 in primary human CD4\(^+\) T cells.

We next investigated whether IL-6 could promote the canonical CXCR5\(^+\) PD1\(^+\)ICOS\(^+\) T\(\text{FH}\) phenotype in vitro. IL-6 failed to induce a significant upregulation of these surface markers in activated CD4\(^+\) T cells (Figure 4a and b, Supplementary Figures S1A and B). As a positive control, we confirmed that IL-12 significantly increased the proportion of cells with the T\(\text{FH}\) phenotype, which is in line with other studies,\(^{21,22}\) whereas IL-21, like IL-6, only marginally increased T\(\text{FH}\) surface marker expression (Figure 4a and b). In confirmation of our mRNA data, however, IL-6 increased intracellular BCL6 expression in activated CXCR5\(^+\) CD4\(^+\) T cells (Figure 4c), but to a lesser extent than IL-12. We also sought to confirm and extend our ELISA data by intracellular flow cytometric analysis of IL-21 in CXCR5\(^+\) cells. Upon FMA/ionomycin treatment to elicit cytokine production, BCL6 expression in CXCR5\(^+\) CD4\(^+\) T cells was uniformly increased as expected,\(^{30}\) but IL-6, IL-12 and IL-21-stimulated cells exhibited a...
specific increase in the proportion of BCL6⁺ IL-21⁺ cells (Figure 4d and e). Normalization to no cytokine (−) control revealed significant effects on the proportion of BCL6⁺ IL-21⁺ cells by culture with IL-6, IL-12 or IL-21 (Figure 4f). In agreement with other studies,21,31 these data show the potency of non-T cell-derived cytokines, namely IL-6 and IL-12, in the induction of IL-21 production by human CD4⁺ T cells.

BCL6 has been implicated in the regulation of TFH differentiation,17,18,20 and sustained CXCR5 is a key marker for TFH cells in both mouse and human.1,2,20,21,32 As IL-6 triggered the upregulation of BCL6 and T-dependent Ig production by the induction of IL-21, we hypothesized that BCL6 expression may enhance CXCR5 expression on human CD4⁺ T cells. To address this question, we retrovirally overexpressed BCL6 in total CD4⁺ T cells (Figure 5a), cultured transduced cells on autologous irradiated feeder cells in the presence of PHA and IL-2, and monitored CXCR5 surface expression (Figure 5b). Unlike its positive effect on long-term expansion in human B cells,33 overexpression of BCL6 did not have this effect on transduced CD4⁺ T cells (Supplementary Figure S2A). In agreement with previous results,34 CXCR5 expression rapidly increased after culture in vitro (Figure 5b). In transduced (nerve growth factor receptor [NGFR]-gated) cells, BCL6 caused a transient, but
associated with trafficking between B and T cell zones and T cell
examined CD80 expression,\(^3\) which was significantly increased in
for the heightened activation status of BCL6-expressing cells, we
fluorescence (see Supplemental Figure S1 for representative FACS plots). Frequencies were determined from isotype control stainings using
a sample pooled from the shown experiments). Quantitation of CXCR5\(^+\) cells within the CD3\(^+\)CD4\(^+\)-gated cells (a) and ICOS/PD1 (b) expression on
CXCR5\(^+\)CD4\(^+\)-gated T cells is shown. (c) Intracellular BCL6 expression in CD3/CD28-activated CXCR5\(^+\)CD4\(^+\)-gated cells. Immediately after culture, cells were
surface stained, fixed, permeabilized and stained for intracellular BCL6. Number on graph indicates percent BCL6-positive cells. Dashed histogram is
isotype control. (d) For intracellular IL-21 detection, CD3/CD28-stimulated cells were washed and restimulated with PMA and ionomycin in the presence of
monensin for 6 h, surface stained, fixed, permeabilized and stained for intracellular BCL6 and IL-21. Isotype control staining was used to determine
frequencies. (e) Frequency of IL-21\(^+\) BCL6\(^+\) cells (gated on CXCR5\(^+\)CD4\(^+\) cells) and (f) and the fold difference in IL-21\(^+\)BCL6\(^+\) cells as compared with no
cytokine control. Graphical results show mean values and are derived from 7 donors in two separate experiments, and FACS plots are representative.
Statistical significance across donors (*, \(P<0.05\); **, \(P<0.01\); #, \(P=0.06\) versus no cytokine control) was determined by pairwise Mann-Whitney tests.

IL-21 potently activates STAT3 in human B cells.\(^{10,11}\) B cell-specific
loss of Stat3 in mice leads to impaired Ab responses\(^{37}\) and mutation of
Stat3 in humans leads to dysregulated humoral immunity.\(^{11,38}\)
We, therefore, directly tested whether upregulation of PRDM1 and IL-6
production by IL-21 in B cells were dependent upon STAT3. To address this, we utilized stable retroviral overexpression of small
hairpin RNA (shRNA)-targeting STAT3. We first tested the STAT3
shRNA efficacy in Raji B cells expressing endogenous STAT3
(Figure 7a). We transduced cells with control shRNA-targeting firefly
Renilla luciferase or STAT3 shRNA, purified transduced green
fluorescent protein (GFP\(^+\)) cells by flow cytometry and determined
STAT3 protein levels. STAT3 shRNA reduced STAT3 protein levels by
~90% (Figure 7a). We then assessed the requirement for STAT3 in
the induction of PRDM1 gene expression by IL-21 in normal primary
human B cells. Total PB CD19\(^+\) cells were transduced with either
shRNA-targeting firefly Renilla luciferase or shSTAT3, GFP-sorted and
cultured in the presence of IL-21 for 3 days. PRDM1 gene
expression was upregulated by IL-21 in cells expressing control
Enforced BCL6 expression enhances lymphoid/follicular markers, including CXCR5 in primary human CD4⁺ T cells. Total CD4⁺ T cells from peripheral blood were stimulated with CD3/CD28 beads for 4 days and transduced (a) with retroviral vectors encoding either LZRS-IRES-ΔNGFR (Control) or LZRS-BCL6-IRES-ΔNGFR (BCL6). (b) CXCR5 surface expression on resting, activated (4 days), or Control and BCL6-transduced CD4⁺ T cells at the indicated time points after transduction. Transduced cells were cultured on irradiated allogeneic PBMC feeders with PHA (2 μg ml⁻¹) and IL-2 (20 U ml⁻¹) (methods). (c) Percent CXCR5⁺ and (d) CXCR5 mean fluorescence intensity of CD4⁺ NGFR⁺ cells over time. (e) CCR7/CXCR4 expression in CD4⁺ NGFR⁺ cells at days 14 and 21 after transduction. (f) CD80 expression on CD4⁺ NGFR⁺ cells 21 days after transduction. For e and f, gates were set using isotype control stainings and total tonsil cells to define positive populations, as described for CXCR4 and CD80⁶¹. CCR7 gates in e were defined as described⁶². Graphical results are means ± s.d. of two independent experiments each containing two donors and FACS plots are representative. Two-way ANOVA was used to calculate statistical significance (P-values shown on graphs) in c and d. Quantitated data for CXCR4/CCR7 and CD80 are shown in Supplementary Figure S2.
students' statistical significance (**, P < 0.01) was determined using unpaired Student’s t-tests.

We then tested the requirement for STAT3 in IL-21-induced IgM and IgG production by both naïve (IgD–CD27+) and total CD19+ B cells from peripheral blood. IL-21 promoted IgM (Figure 7c) and IgG (Figure 7d) production by naïve B cells that was sharply reduced in cells transduced with shSTAT3. In total, CD19+ cells (containing 71 ± 5% IgD–CD27+ naïve B cells and 7 ± 3% IgD+CD27+ memory B cells), IL-21 also induced IgM (Figure 7c) and IgG (Figure 7d) production in control-transduced B cells. Similar to the effect seen in naïve B cells, shSTAT3-transduced total B cells also exhibited reduced IgM production in response to IL-21 (Figure 7e). Although IL-21 induced modest IgG production in shSTAT3-transduced total CD19+ B cells (Figure 7f), these levels were still significantly reduced compared with those made by IL-21-treated shRNA-targeting firefly Renilla luciferase-transduced total CD19+ B cells (Figure 7f). Together, these results demonstrate that IL-21 requires STAT3 to promote plasma cell differentiation and optimal Ig production in human B cells.

DISCUSSION

Here, we have investigated the molecular requirements for human T cell: B cell collaboration in the induction of Ab production. Non-T cell-derived cytokines such as IL-6 and IL-12 stimulated IL-21 production by CD4+ T cells, with or without acquisition of a canonical surface TFH phenotype. IL-12 promoted expression of CXCR5, ICOS, PD-1, BCL6 and IL-21 in activated CD4+ T cells. Although IL-6 failed to affect CXCR5, ICOS or PD-1 expression, this cytokine significantly increased BCL6 and IL-21 production and perpetuated Ig production by B cells. Nonetheless, retroviral overexpression of BCL6 itself triggered only a brief upregulation of CXCR5, rather than the stable reprogramming observed in murine CD4+ T cells.17 Although multiple stimuli induce IL-21 production and contribute to the TFH phenotype, it is clear from our results using stable genetic reduction of STAT3 expression in normal B cells that this transcription factor is required for IL-21-induced B cell differentiation. These results, therefore, provide an important confirmation of previous results obtained in individuals containing rare STAT3 mutations.11

From our results and those from others, it is becoming clear that non-T cell-derived cytokines are important in the induction of IL-21-mediated B cell helper activity by CD4+ T cells. In vitro, IL-12 has been shown to increase IL-21 production by naïve human CD4+ T cells and provide B cell help in an IL-21-dependent manner.21,22 In the mouse, IL-12 does not exert this effect.23 Compared with IL-12, others have shown a less potent effect of IL-6 on IL-21 production in naïve human CD4+ T cells.21 Nonetheless, our results show that the positive effect of IL-6 on IL-21 production by total CD4+ T cells and
provision of B cell help is also dependent on IL-21. As to the in vivo source of IL-21-inducing cytokines, IL-6 and IL-12 are likely to be important, as in the GC these cytokines are produced by dendritic cells\textsuperscript{22,39,40} and IL-6 is produced by follicular dendritic cells.\textsuperscript{41} Thus, it is possible that these cytokines may synergize in vivo to induce IL-21 production and promote effective humoral immunity.

IL-21 itself has also been shown as a differentiation factor for T\textsubscript{FH} cells in the murine system.\textsuperscript{2} We confirmed that IL-21 can autoregulate itself\textsuperscript{42} in total CD4\textsuperscript{+} T cells. As IL-21 is produced predominantly by T cells, it is possible that IL-21 may reinforce T\textsubscript{FH} differentiation upon activation of naive CD4\textsuperscript{+} T cells by APCs. As our studies were performed in total CD4\textsuperscript{+} T cells, it is also possible that memory or differentiating effector CD4\textsuperscript{+} T cells selectively respond to IL-21 and upregulate its own expression. This issue may require further study for two reasons: (1) CXCR5\textsuperscript{+} T\textsubscript{FH} cells are not the only CD4\textsuperscript{+} T cells capable of producing IL-21, and (2) although CD45RO\textsuperscript{+}CXCR5\textsuperscript{+} T\textsubscript{FH} memory-like cells have been described, their ultimate contribution to long-lived humoral immunity has not been clarified.\textsuperscript{5}

Expression of BCL6 in T cells has been shown to be essential for optimal IL-21 production and T\textsubscript{FH} development in vivo.\textsuperscript{15–19} We show here that in accordance with the ability to promote IL-21 secretion, non-T cell-derived cytokines like IL-6 and IL-12 also induce BCL6 in CD4\textsuperscript{+} T cells. IL-21 itself, however, did not induce significant BCL6 expression, although we observed autoregulation of IL-21. This is in contrast to B cells, where IL-21 induces BCL6.\textsuperscript{10,43,44} Perhaps, regulation of BCL6 by IL-21 in CD4\textsuperscript{+} T cells is delayed compared with the effect of IL-6. Moreover, regulation of BCL6 is complex. Cytokine-mediated gene regulation, antigen receptor signaling and CD40L ligation, all modulate BCL6 protein stability and its molecular interactions in B cells.\textsuperscript{45–47} but whether similar mechanisms are at play in CD4\textsuperscript{+} T cells is unknown. We propose that non-T cell-derived cytokines like IL-6 and IL-12 are strong initial inducers of BCL6 and IL-21 expression, and that IL-21 reinforces its own production in a mechanism that may or may not require BCL6. Consistent with this notion is the fact that many different GC-resident cells produce these initiating cytokines in vivo, whereas IL-21 production is restricted mainly to activated T cells.\textsuperscript{48}

Our data here depict a complex mechanism regulating human T\textsubscript{FH} development. On the one hand, our data show that in line with the ability of IL-6 to trigger IL-21 production in CD4\textsuperscript{+} T cells, IL-6 also increased BCL6 mRNA expression. Sustained CXCR5 expression is a key defining T\textsubscript{FH} cell marker.\textsuperscript{1,2} BCL6 overexpression led to a transient upregulation of CXCR5 and a stable increase in other lymphoid/follicle chemokine receptors (CCR7 and CXCR4, respectively) in CD4\textsuperscript{+} T cells. CCR7 expression is present on a proportion of CD4\textsuperscript{+}CXCR5\textsuperscript{+}CD200\textsuperscript{+}T\textsubscript{FH} cells in the tonsil.\textsuperscript{20} Recently, the development of CCR7\textsuperscript{+}CD4\textsuperscript{+} central memory CD4\textsuperscript{+} T cell precursors was shown to require the expression of Bcl6.\textsuperscript{35} We also note the increased expression of CD80 on BCL6-transduced CD4\textsuperscript{+} T cells. On the contrary, in GC B cells, BCL6 has been shown to downregulate CD80 expression.\textsuperscript{49} CD80 expression on mouse T cells has been proposed to have an immunoregulatory role,\textsuperscript{50} but high CD80 expression on murine T\textsubscript{FH} cells has also been reported.\textsuperscript{51} CD80 expression on human CD4\textsuperscript{+} T cells is thought to be immunostimulatory.\textsuperscript{56} Given that CD80 is a ligand in the PD-1/PD-L1\textsuperscript{57} pathway and that high PD-1 expression correlates with high human T\textsubscript{FH} function,\textsuperscript{55} it is possible that CD80 expression may help support the function of PD-1\textsuperscript{+} T\textsubscript{FH} cells. Taken together, acquisition of these markers under conditions of high BCL6 expression may, therefore, enable a subset of functional memory precursor CD4\textsuperscript{+} T cells to transit rapidly between T and B cell zones in follicles to promote the humoral response. Indeed, expression of BCL6 has been previously linked to T cell memory.\textsuperscript{54,55}

Our data suggest that sustained BCL6 expression transiently increases CXCR5 expression in CD4\textsuperscript{+} T cells. However, the CXCR5 levels achieved are not as high as those found on bona-fide tonsillar T\textsubscript{FH} cells (not shown). Thus, BCL6 expression may support, but is not solely sufficient for, the acquisition of a CXCR5\textsuperscript{hi} T\textsubscript{FH} phenotype in human CD4\textsuperscript{+} T cells in vitro. Ex vivo-sorted tonsillar CXCR5\textsuperscript{hi} cells are BCL6\textsuperscript{+}, IL-21\textsuperscript{+}, and are efficient B cell helpers.\textsuperscript{20,21} As our culture conditions involved robust T cell stimulation, our results suggest that factors in addition to high BCL6 expression, TCR stimulation and endogenously produced T-cell-derived cytokines are necessary to produce the sustained CXCR5\textsuperscript{hi} T\textsubscript{FH} phenotype in human CD4\textsuperscript{+} T cells in vitro. Further study of CXCR5 gene regulation\textsuperscript{56} may reveal insights into factors necessary to stably convert human BCL6\textsuperscript{hi} CD4\textsuperscript{+} T cells into T\textsubscript{FH} cells.

Irrespective of which factors may trigger IL-21 production, the presence of this cytokine strongly promotes B cell differentiation.\textsuperscript{39} We showed that IL-21 was required for the indirect promotion of Ig production by IL-6 in CD4 T cell: B cell co-cultures. A similar dependence on IL-21 for IL-12-induced Ig production was also shown.\textsuperscript{21,22} Activated human primary B cells upregulate PRDM1 and secrete Ig in response to IL-21.\textsuperscript{10,27} We previously found that specific activation of STAT3 induced plasma cell differentiation and Ig secretion to levels similar to those induced by IL-21.\textsuperscript{10,11} These results suggested, but did not demonstrate, a requirement for STAT3 in the induction of plasma cell differentiation and Ab secretion. Here, we now show that the induction of PRDM1 by IL-21 requires activation of STAT3. Downregulation of STAT3 levels also led to a significantly blunted ability of B cells to produce IgM or IgG in response to IL-21. Our data indicate that both naive and switched memory B cells require STAT3 for optimal Ab production in response to IL-21. Our results using normal cells from healthy donors are in concordance with findings in patients with inactivating STAT3 mutations, wherein B cell responses to IL-21 are severely diminished.\textsuperscript{11} Together, these data demonstrate the critical importance of STAT3 activation for efficient generation of high-level Ab secretion by and its role as a critical molecular hub in the integration of plasma cell differentiation and production of both switched and non-switched Ig.

In summary, our data provide mechanistic insight into how human CD4\textsuperscript{+} T cells may respond to non-T cell-derived cytokines in order to promote humoral immunity by the IL-21/STAT3 axis. IL-6 and IL-12 triggered IL-21 production by CD4\textsuperscript{+} T cells, and IL-21 in turn induced PRDM1 gene expression and Ig production by B cells in a STAT3-dependent manner. IL-6 and IL-12 also upregulated expression of BCL6 in activated CD4\textsuperscript{+} T cells, which is associated with the production of IL-21 and a T\textsubscript{FH} phenotype.\textsuperscript{21} These results also posit that non-T cell-derived cytokines may synergize to drive IL-21 production in vivo. Our overexpression studies indicate that high BCL6 levels may serve as a platform for the acquisition of the T\textsubscript{FH} phenotype in humans, and suggest that additional cellular factors such as costimulation\textsuperscript{57,58} may be required to fully drive the T\textsubscript{FH} phenotype. Deciphering the signals required to manifest and maintain the T\textsubscript{FH} phenotype will be important for understanding humoral immunity and for vaccine development.

**METHODS**

**Human cell isolation**

Peripheral blood mononuclear cells (PBMCs) were obtained from leukofilters prepared from adult peripheral blood (Sanguin Blood bank, Amsterdam, the Netherlands) or from venipuncture and separation by Ficoll–Paque gradients.
B cell cultures: 5 x 10^6 B cells were cultured with PHA (HA16, 2 μg ml⁻¹, Sigma-Aldrich, St Louis, MO, USA) in a 1 ml culture in 24-well plates (Costar, Corning, NY, USA). Cells were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM, Invitrogen, Grand Island, NY, USA) supplemented with 8% FCS (HyClone, Hyclone-Thermo Fisher Scientific, Rockford, IL, USA) and penicillin/streptomycin (Roche Applied Science, Indianapolis, IN, USA).

Flow cytometry analysis
The following monoclonal antibodies (mAbs) against the human molecules CD3 (SK7), CD4 (RPA-T4), CD19 (4G7, SJ25C1, ID3 or HB19), CD20 (2H7), CD138 (MI15) were obtained from Dako (Carpenteria, CA, USA). Anti-NGFR retroviral vectors have been described. Gibbon ape leukemia virus (GALV)-pseudotyped retroviruses were produced using the Phoenix GalV packaging cell line (a kind gift of Garry Nolan, Stanford University, Stanford, CA, USA).

Enzyme-linked immunosorbent assay
Plates were coated with capture Abs anti-human IgG, IgM or IgA (Dako) at 5 μg ml⁻¹ in 0.1M NaHCO₃ pH 9.6 for 2 h at 37°C and washed in 0.1M NaHCO₃ pH 9.6 for 2 h at 37°C and washed in 0.1M NaHCO₃ pH 9.6. Plates were coated with capture Abs anti-human IgG, IgM or IgA (Dako) at 5 μg ml⁻¹ in 0.1M NaHCO₃ pH 9.6 for 2 h at 37°C and washed in 0.1M NaHCO₃ pH 9.6.
CONFLICT OF THE INTEREST
The authors declare no conflict of interest.

ACKNOWLEDGEMENTS
This work was supported by NIH Grants AI063846 (to SAD) and by the National Center for Research Resources and the National Institute of General Medical Sciences of the NIH through Grant Numbers P20RR019050 and P30GM103532. We thank Berend Hooibrink and Colette Charland of the Academic Medical Center and University of Vermont flow cytometry core facilities, respectively, for cell sorting and equipment maintenance. We thank Dr Oliver Dienz for critical reading of the manuscript.

Author contributions: SAD, H Schmidlin and MN performed experiments. BB and H Spits analyzed data and provided valuable intellectual input. SAD conceived and directed the project and wrote the paper (with input from the other authors).

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