Cytokine-Mediated Regulation of Human B Cell Differentiation into Ig-Secreting Cells: Predominant Role of IL-21 Produced by CXCR5$^+$ T Follicular Helper Cells[1]

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Differentiation of B cells into Ig-secreting cells (ISC) is critical for the generation of protective humoral immune responses. Because of the important role played by secreted Ig in host protection against infection, it is necessary to identify molecules that control B cell differentiation. Recently, IL-21 was reported to generate ISC from activated human B cells. In this study, we examined the effects of IL-21 on the differentiation of all human mature B cell subsets—neonatal, transitional, naive, germinal center, IgM-memory, and isotype-switched memory cells—into ISC and compared its efficacy to that of IL-10, a well-known mediator of human B cell differentiation. IL-21 rapidly induced the generation of ISC and the secretion of vast quantities IgM, IgG and IgA from all of these B cell subsets. Its effect exceeded that of IL-10 by up to 100-fold, highlighting the potency of IL-21 as a B cell differentiation factor. Strikingly, IL-4 suppressed the stimulatory effects of IL-21 on naive B cells by reducing the expression of B-lymphocyte induced maturation protein-1 (Blimp-1). In contrast, memory B cells were resistant to the inhibitory effects of IL-4. Finally, the ability of human tonsillar CD4$^+$CXCR5$^+$CCR7$^-$ T follicular helper (T_{FH}) cells, known to be a rich source of IL-21, to induce the differentiation of autologous B cells into ISC was mediated by the production of IL-21. These findings suggest that IL-21 produced by T_{FH} cells during the primary as well as the subsequent responses to T cell-dependent Ag makes a major contribution to eliciting and maintaining long-lived humoral immunity. *The Journal of Immunology,* **2007, 179: 8180–8190.**

The differentiation of mature B cells into plasma cells (PC)[5] is critical for the generation of protective humoral immune responses. Indeed, the efficient production of Ag-specific Ig by activated B cells underlies the success of most vaccines (1, 2). Many cytokines can induce Ig secretion by human B cells activated with mimics of T cell-dependent (i.e., CD40L) or T cell-independent (i.e., engaging the BCR) stimuli in vitro. These include IL-2 (3–6), IL-4 (5, 7–9), IL-6 (3, 10, 11), IL-10 (3, 5, 12–14), IL-12 (15), IL-13 (16), IL-15 (17), and TGF-β (3, 18). Many of these cytokines have different efficacies and specificities. For instance, their effects often favor the production of a particular Ig isotype(s), with IL-4 and IL-13 inducing IgG4 and IgE secretion (16), TGF-β directing production of IgA (18), and IL-2, IL-10, and IL-15 increasing secretion of IgM, IgG1, and IgA (5, 13, 17). IL-4 has a greater effect on naive than on germinal center (GC) and memory B cells (19), while IL-10 preferentially enhances differentiation of GC and memory B cells into Ig-secreting cells (ISC) (20–24). Similarly, IL-6 exerts its effect on preactivated B cells (10, 11), suggesting that it acts at a later stage of B cell differentiation than other cytokines. Despite these findings regarding the activity of numerous cytokines on B cell activation, the in vitro effect of IL-10 exceeds that of other cytokines by up to 100-fold (5, 12, 13, 17), making it a well-recognized and potent inducer of Ig secretion by human B cells.

Over the past few years, IL-21 has emerged as a strong inducer of human B cell differentiation (25–28). Specifically, IL-21 has been found to induce human naive splenic B cells to undergo isotype switching to produce IgG1 and IgG3 (26) and to induce cord blood (CB) and peripheral blood (PB) B cells to differentiate into PC (28). Although this latter study unequivocally demonstrated the ability of IL-21 to mediate the differentiation of human B cells into ISC (28), it did not compare the effects of IL-21 to other cytokines such as IL-10, nor did it compare the effects of IL-21 on naive and memory B cells from the same tissue (28). Furthermore, no information has been presented detailing the sensitivity of GC B cells to the differentiative effects of IL-21. For these reasons, we quantitatively examined the effects of IL-21 on the differentiation of all subsets of human mature B cells—neonatal, transitional, naive, GC, IgM memory, and isotype switched memory—into ISC and

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[5] Abbreviations used in this paper: PC, plasma cells; AITL, angioimmunoblastic T-cell lymphoma; Blimp-1, B lymphocyte-induced maturation protein-1; CB, cord blood; GC, germinal center; ISC, Ig-secreting cell; PB, peripheral blood; T_{FH}, T follicular helper.

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compared its efficacy to that of IL-10. Because $CD4^+CXCR5^+CCR7^-$T follicular helper (T_{FH}) cells present in lymphoid tissues are a prominent source of IL-21 (29–32), we also examined the contribution of T_{FH} cell-derived IL-21 to B cell differentiation in vitro.

Materials and Methods

Abs and Reagents

The following Abs were used: FITC-anti-CD20, FITC-anti-CD57, PE-Texas Red-anti-CD4, and allophycocyanin-anti-CD38 mAb (BD Biosciences); PE-anti-CD27, biotinylated anti-IgM, IgG, and IgA mAb, Alexa 647-anti CXCR5, and streptavidin (SA) conjugated to PerCp (BD Pharmingen); PE-anti-CD38 (Caltag); and biotinylated and allophycocyanin-anti-CD27 mAb (eBioscience). Membranes of insect cells infected with baculovirus expressing recombinant human CD40L were prepared by Dr. G. Shoebridge and N. Hare (Centenary Institute of Cancer Medicine and Cell Biology, Newtown, New South Wales, Australia).

Human B cells

Human spleens from cadaveric organ donors and buffy coats from healthy donors were obtained from the Australian Red Cross Blood Service (Sydney, Australia). Tonsils and CB samples were provided by the Royal Prince Alfred Hospital (Sydney, Australia). Institutional human ethics review committees approved all studies. Transitional, naïve, and memory B cells were isolated from PB by sorting $CD20^+CD10^+CD27^-$, $CD20^+CD10^-CD27^-$, and $CD20^+CD10^-CD27^+$ cells, respectively (24, 33–35). Splenic naive, IgM memory, and isotype-switched memory B cells were isolated by sorting $CD20^+CD27^-IgG/A/E^-$, $CD20^+CD27^+IgG/A/E^-$, and $CD20^+CD27^+IgM/D^-$ cells, respectively (23, 24, 35–37). GC B cells were sorted as $CD20^{high}CD38^{high}$ cells from tonsil mononuclear cells (20, 38). Cells were sorted on a FACSVantage or Aria flow cytometer (BD Immunohistochemistry Systems); postsort purity was >98%.

B cell cultures

Purified B cells were cultured in B cell medium (RPMI 1640 containing L-glutamine (Invitrogen Life Technologies), 10% FCS (Invitrogen Life Technologies), 10 mM HEPES (pH 7.4) (Sigma-Aldrich), 0.1 mM nonessential amino acid solution (Sigma-Aldrich), 1 mM sodium pyruvate (Invitrogen Life Technologies), 60 mg/ml penicillin, 100 mg/ml streptomycin, 40 mg/ml transferrin (Sigma-Aldrich), and 20 μg/ml Normocin (InVivogen); Refs. 23 and 24) and stimulated with CD40L alone or with IL-4 (100 U/ml), IL-10 (50 U/ml; both provided by DNAX Research Institute, Palo Alto, CA), IL-21 (50 ng/ml; Peprotech), or IL-4 and IL-21. For some experiments, B cells were labeled with CFSE (Molecular Probes) (24). For phenotypic and functional analysis, the cells were cultured in 48-well plates (2×10^5/500 μl/well; Becton Dickinson Labware) for 4 or 5 days. In some experiments, B cells were cultured in 96-well plates (50×10^3 cells per 200 μl per well; Becton Dickinson Labware) for 10–12 days. For the data presented in Fig. 4, naive and memory B cells were sorted from human spleens as $CD19^+CD27^-$ and $CD19^+CD27^+$ cells (35), respectively, and then cultured (10×10^3 cells per 200 μl) with irradiated CD32 (FcγRII)-transfected L cells (20×10^3 per 200 μl) and anti-CD40 mAb (mAb89) (8, 12, 18) in the presence or absence of IL-21 (Cell Sciences), IL-2 (100 U/ml; R&D Systems), or IL-10 for 10 days in Yssel's medium (35).

Assessing B cell differentiation into ISC

Phenotypic analysis. In vitro activated CB, naive, memory, or GC B cells were harvested after 4 days of culture and then incubated with PE-anti-CD27 and allophycocyanin-anti-CD38 mAb. The samples were fixed in 1% formaldehyde; data were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo (Tree Star). The frequency of cells expressing high levels of CD38 and CD27 (i.e., $CD38^{high}CD27^{high}$; Ref. 39) and the percentage of $CD38^{high}CD27^{high}$ cells in each division were determined (24). The absolute number of cells was calculated by adding a known number of CaliBRITE beads (BD Biosciences) to each well before harvest (24).

ELISPOT analysis. Activated B cells were harvested after 4 days of in vitro culture and then incubated in the wells of MultiScreen-HA ELISPOT plates (Millipore) precoated with goat anti-human IgM, IgG, and IgA polyclonal antisera (Southern Biotech). The percentages of cells secreting IgM, IgG, and IgA were determined as previously described (24, 39, 40).

ELISA. Secretion of IgM, IgG, and IgA was determined by Ig H chain-specific immunoassays (12, 18, 24, 35).

$CD4^+$ T cell/B cell cocultures

$CD4^+$ T cells were isolated from human tonsils using CD4 Dynabeads (Dynal) (41). $CD4^+CXCR5^+CD57^{+/-}$ and $CXCR5^-$ subsets were isolated by cell sorting, treated with mitomycin C (40 μg/ml; Sigma-Aldrich) for 1 h at room temperature, and then cultured in 96-well U-bottom tissue culture plates with autologous B cells (25×10^3 cells per 200 μl per well) alone (41) or with PHA (5 μg/ml; Sigma-Aldrich) and IL-2 (20 U/ml; Chemicon) in the absence or presence of human IgG1 (Calbiochem) or IL-21R-Fc (10 μg/ml; R & D Systems). Ig secretion was determined after 5–10 days by ELISA.

Semiquantitative PCR analysis

RNA was isolated from activated B cells (Qiagen RNeasy kit; Qiagen) and then transcribed into cDNA using random hexamers (Invitrogen Life Technologies) as primers and Superscript II RNase H^- reverse transcriptase (Invitrogen). The resulting cDNA was normalized for expression of the constitutively expressed gene GAPDH (TGGTCGTATTGGGCGC (5′) and GGTCATGAGTCCTTCACGATACC (3′)) and then used as a template for PCR. The following primers were used (Sigma-Genosys): Pax-5 (GCATAGTGTCCACTGGCTCC (5′) and CCAGGAGTCGTTGTAC GAGG (3′)) and B-lymphocyte induced maturation protein-1 (Blimp-1) (GATGCGGATATGACTCTGTGG (5′) and CTCGGTTGCTTTAGAC TGCTC (3′)) (39, 40).

Western blotting

Naive and memory B cells were cultured in vitro for 4 days and then solubilized in ice-cold lysis buffer (10 mM Tris-HCl (pH 7.8), 1% Nonidet P-40, 150 mM NaCl, and enzyme inhibitors; Refs. 39 and 42). Cell lysates were electrophoresed through 12% acrylamide gels containing 0.1% SDS and transferred to PVDF membranes (Millipore). Membranes were probed with Abs against Blimp-1 (39, 43), Pax-5 (C-20) or SHP-2 followed by HRP-conjugated anti-rat, goat, or rabbit Ig antiserum, respectively (all from Santa-Cruz Biotechnology). The membranes were developed using ECL (Pierce).

Results

IL-21 potently induces division-linked differentiation of human naive and memory B cells into ISC

Because IL-21 has been reported to induce CB B cells and PB memory B cells to generate ISC in vitro (28), we were interested in determining the relative rates of differentiation of distinct subsets of human B cells. Naive CB or splenic B cells yielded <1% $CD38^{high}CD27^{high}$ ISC following stimulation for 4 days with CD40L with or without IL-10 (Fig. 1, *a* and *b*). In contrast, similar frequencies (~6%) of $CD38^{high}CD27^{high}$ cells were detected in cultures of either splenic IgM memory or switched memory cells stimulated with CD40L/IL-10 (Fig. 1, *c* and *d*). Strikingly, IL-21 dramatically increased the frequency of $CD38^{high}CD27^{high}$ cells generated in cultures of CB, naive, and memory subsets by 5- to 20-fold compared with that induced by CD40L alone or CD40L/IL-10 (Fig. 1).

Differentiation of human memory B cells into $CD38^{high}CD27^{high}$ ISC induced by CD40L/IL-10 is linked to cell division (23, 24, 39). When CFSE-labeled B cells were examined, IL-21-induced differentiation of naive and memory cells into ISC was also found to be division linked (Fig. 1, *a* and *b*, *far right panel*). This contrasted the effect of IL-10, which yielded substantially fewer ISC irrespective of division history (Fig. 1, *a* and *b*). Although both IL-10 and IL-21 induced CD40L-stimulated IgM memory and switched memory B cells to become ISC, their rate of differentiation was much greater when cultured with IL-21 (Fig. 1, *c* and *d*; *right panel*). Thus, both memory B cell subsets underwent more than two divisions before they became ISC in response to CD40L/IL-10, whereas a small fraction (~5%) of ISC were generated from CD40L/IL-21-stimulated memory B cells before they underwent their first division (Fig. 1, *c* and *d*; *far right panel*). Once CD40L/IL-21-stimulated memory B cells had entered division, more of them became ISC with each subsequent division than

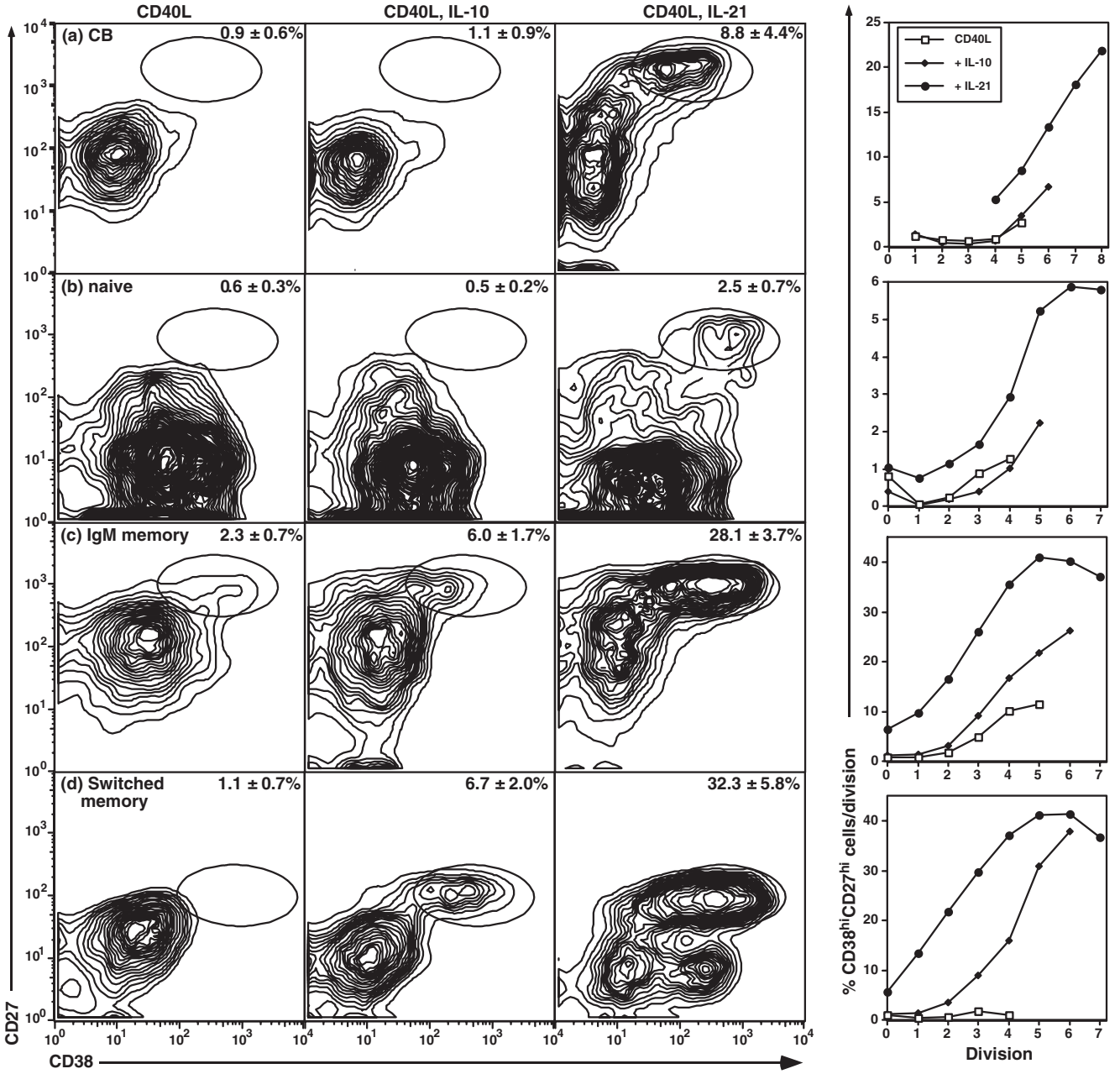

FIGURE 1. IL-21 potently induces the division-linked differentiation of naive and memory B cells into effector cells with an ISC phenotype. CB B cells (*a*) or splenic naïve (*b*), IgM memory (*c*), or Ig isotype-switched memory B (*d*) cells were cultured with CD40L alone or together with IL-10 or IL-21. The cells were harvested after 4 days and the frequency of cells with a $CD38^{high}CD27^{high}$ phenotype was determined. The values represent the mean ± SEM of four (*a*), five (*b* and *c*), and two (*d*) independent experiments. In some experiments, B cells were CFSE-labeled before culture and the frequency of $CD38^{high}CD27^{high}$ cells per division was determined by division slicing. Note, there are no values in *a* for B cells in divisions 0–3 in cultures containing IL-21 because most of them had undergone more than four divisions (see Ref. 37).

those stimulated with CD40L/IL-10 (Fig. 1, *c* and *d*). The division-linked rate of differentiation of IgM memory and isotype-switched memory cells in response to CD40L/IL-21 greatly exceeded that of naive B cells (Fig. 1, *b–d*). Specifically, only 5–6% of ISC were generated from naive B cells that had undergone the greatest number of cell divisions (Fig. 1*b*, *right panel*), while ~40% of both memory B cell populations in these same divisions were ISC (Fig. 1, *c* and *d*; *right panel*). The differences in the rate of generation of ISC from stimulated splenic naive and memory B cells on a division basis mirrored differences in the frequency of ISC generated in the bulk population (compare Fig. 1, *b–d*).

The generation of functional ISC was assessed using ELISPOT by determining the frequency of cells secreting IgM, IgG, and IgA. Overall, the frequencies of functional ISC correlated with $CD38^{high}CD27^{high}$ cells (Table I). Although most ISC generated from naive populations produced IgM, low frequencies of IgG and IgA ISC were also detected, demonstrating that IL-21 can facilitate isotype switching not only to IgG (26, 28), but also to IgA. IgM memory B cells yielded cells secreting IgM, and a significant proportion (>50%) also secreted IgG or IgA (Table I), thus revealing a heightened ability of IgM memory cells to undergo isotype switching compared with naive cells. Switched memory B cells predominantly secreted IgG and IgA (Table I), consistent with the acquisition of these isotypes in vivo. Thus, IL-21 is more potent than IL-10 at inducing the differentiation of human CD40L-stimulated B cells into ISC, mediating the

Table I. *IL-21 induces differentiation of human B cells into functional ISC more efficiently than IL-10*[a]

B cells	Stimulation	Percentage Ig-Secreting Cells		
		IgM	IgG	IgA
Cord blood	CD40L	0.2	0.0	0.0
	+ IL-10	0.6	0.1	0.0
	+ IL-21	10.4	0.1	0.7
Naive	CD40L	0.0	0.05	0
	+ IL-10	0.15	0	0.05
	+ IL-21	2.1	0.5	0.25
IgM-memory	CD40L	0.1	0.2	0.0
	+ IL-10	1.2	1.1	0.2
	+ IL-21	6.2	10.5	1.1
Switched memory	CD40L	0.1	0.25	0.1
	+ IL-10	0.125	1.13	1.7
	+ IL-21	0.7	7.4	12.0

[a] CB B cells or splenic naive, IgM memory, and Ig isotype-switched memory B cells were cultured with CD40L alone or together with (+) IL-10 or IL-21. The cells were harvested after 4 days and the frequency of cells secreting IgM, IgG, and IgA was determined by ELISPOT.

differentiation of both memory B cell types and some naive B cells, which are less responsive to the effects of IL-10.

Kinetics of IL-21-induced Ig secretion by CD40L-stimulated human B cells

The kinetics and magnitude of Ig secretion by naive and memory B cell subsets were next examined. After 2 days of stimulation with CD40L/IL-21, naive splenic B cells secreted ~10- and ~5-fold more IgM than those stimulated with CD40L alone and CD40L/IL-10, respectively (Fig. 2*a*, *left panel*). As the culture continued, CD40L/IL-21-induced IgM secretion dramatically increased and exceeded that observed with CD40L/IL-10 by 10–50-fold, depending on the day of analysis. Secretion of IgG and IgA by naive B cells was not detectable at early time points for any of the cultures (Fig. 2*a*, *middle* and *right panels*). However, CD40L/IL-21 induced secretion of switched Ig isotypes by naive B cells within 3–4 days of culture, at least 24 h earlier than CD40L/IL-10. Naive B cells secreted 30–50-fold more IgG and IgA following stimulation with CD40L/IL-21 than with CD40L/IL-10 (Fig. 2*a*, *middle* and *right panels*). These findings were also reflected in an analysis of IgM memory and isotype-switched memory B cells, in that CD40L/IL-21 induced the secretion of all Ig isotypes examined more rapidly and to a greater extent (10- to 200-fold) than CD40L/IL-10 (Fig. 2, *b* and *c*, *right panels*). When the production of Ig isotypes by transitional, naive, and memory PB B cells stimulated with CD40L and either IL-10 or IL-21 was assessed, it was also apparent that the effect of IL-21 exceeded that of IL-10 by 10–100-fold and that these effects were clearly measurable after 10–12 days of in vitro culture (Table II). Overall, this kinetic analysis revealed IL-21 as a potent inducer of Ig production by naive and memory B cells and demonstrated for the first time that IL-21 induces substantial production of IgA, in addition to IgM and IgG, by these subsets of human B cells.

GC B cells robustly respond to IL-21

A predominant source of IL-21 is the T_{FH} cell (29, 32). T_{FH} cells express CXCR5 and consequently home to the B cell areas of lymphoid tissues where they reside in close proximity to GC B cells (31, 44). Thus, it is likely that GC B cells would be targets for IL-21 produced by T_{FH} cells. Tonsil GC B cells were cultured for 4 days with CD40L, CD40L/IL-10, or CD40L/IL-21 to examine the relative effects of these cytokines on the generation of ISC. Quantitation of ISC by flow cytometry and ELISPOT yielded comparable results, with <1, ~4, and ~20% of cells, respectively, harvested from cultures of GC B cells

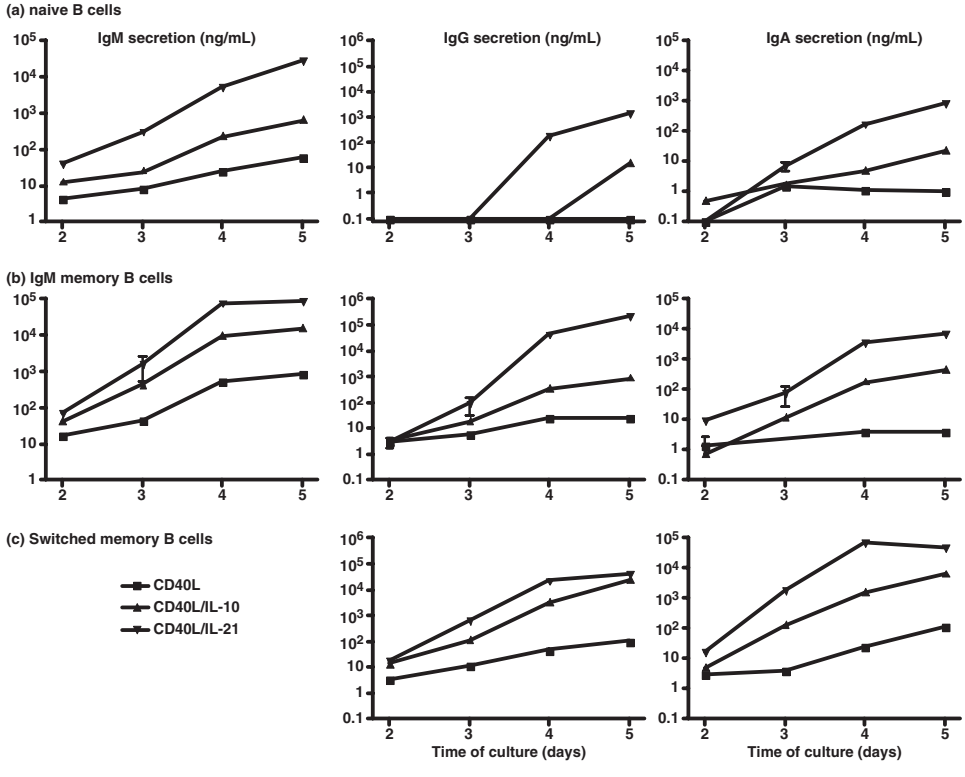

FIGURE 2. Kinetics of Ig secretion induced by IL-10 and IL-21 from CD40L-stimulated human B cells. Naive (*a*), IgM memory (*b*), and switched memory B (*c*) cells from the same donor spleen were cultured with CD40L alone (■) or together with IL-10 (▲) or IL-21 (▼). Supernatants were harvested after 2, 3, 4, and 5 days of culture and the levels of secreted IgM (*left panels*), IgG (*middle panels*), and IgA (*right panels*) were determined by ELISA. Note that the scale on the *y*-axis for each Ig isotype secreted by the different B cell subsets is the same. IgM secretion by switched memory B cells is not presented, as these cells produce only small amounts of this isotype.

Table II. *IL-4 reduces IL-21-induced differentiation of transitional and naïve, but not memory, B cells*[a]

	Ig Secretion (μg/ml)								
	Transitional			Naive			Memory		
	IgM	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA
CD40L	<0.1	<0.01	<0.01	<0.1	<0.01	<0.01	0.2 ± 0.08	0.02	0.03
+ IL-4	0.12 ± 0.07	<0.01	<0.01	0.1 ± 0.07	<0.01	<0.01	<0.1	0.04	0.07
+ IL-10	0.6 ± 0.28	<0.01	<0.01	1.71 ± 0.9	0.026 ± 0.01	0.02 ± 0.01	9.5 ± 2.4	2.1 ± 0.4	3.1 ± 1.0
+ IL-21	65.4 ± 17.4	6.4 ± 2.3	2.4 ± 1.0	105.2 ± 27.3	10.2 ± 5.2	2.3 ± 1.1	85.3 ± 9.8	25.2 ± 5.7	55.0 ± 15.5
+ IL-4 + IL-21	17.1 ± 6.5	4.0 ± 1.7	0.5 ± 0.25	17.9 ± 7.0	2.1 ± 1.0	0.68 ± 0.1	66.4 ± 22.0	25.0 ± 2.7	50.6 ± 10.5

[a] Transitional, naïve and memory B cells were isolated from PB and then cultured with CD40L alone or together with (+) IL-4, IL-10, IL-21, or IL-4 and IL-21. After 10–12 days, secretion of IgM, IgG, and IgA was determined. The values are the means ± SEM of four independent experiments.

stimulated with CD40L, CD40L/IL-10, and CD40L/IL-21 being ISC (Fig. 3, *a* and *b*). IL-21 improves the survival of CD40L-stimulated GC B cells, increasing the recovery of viable cells by ~5-fold compared with CD40L alone (37). Thus, when the total number of ISC generated was determined, CD40L/IL-21 stimulated the generation of ~200- and ~20-fold more ISC than did CD40L alone and CD40L/IL-10, respectively (Fig. 3*c*). The ability of IL-21 to generate large numbers of ISC from CD40L-stimulated GC B cells was also reflected in the secretion of extraordinarily high levels of all Ig isotypes by these cells (Fig. 3*d*). Thus, GC B cells are highly responsive to the stimulatory effects of IL-21.

IL-10 and IL-2 reduce the dose of IL-21 required to induce Ig secretion by human B cells

We next questioned whether the effect of IL-21 on Ig secretion by CD40L-activated B cells could be augmented by adding IL-10 alone or together with IL-2. These cytokines were chosen because IL-10 can induce the differentiation of memory B cells into ISC, and IL-2 enhances the response of CD40L/IL-10-stimulated B cells (23, 24, 45). When IL-21 was used at a saturating concentration (i.e., 50–100 ng/ml), the addition of IL-10 and/or IL-2 did not further increase Ig secretion (data not shown). For this reason, the effects of IL-2 and IL-10 tested in conjunction used with suboptimal doses of IL-21. CD40-stimulated naive B cells responded to IL-21 at concentrations >1 ng/ml as evidenced by the production of IgM and IgG (Fig. 4, *a*, *b*, *d*, and *e*). In contrast, CD40-stimulated memory B cells exhibited 10-fold greater sensitivity to IL-21 (Fig. 4, *c* and *f*). The effect of adding IL-10 to naive B cells stimulated through CD40 and with increasing concentrations of IL-21 was twofold. First, it increased IgM and IgG secretion induced by 1 ng/ml IL-21 by 2- to 5-fold (Fig. 4, *a* and *b*). Second, it reduced the concentration of IL-21 necessary for IgG secretion by 10-fold (Fig. 4*b*). IL-10 also increased IgG production by memory B cells stimulated with 0.1–1 ng/ml IL-21 (Fig. 4*c*). The combination of IL-2 and IL-10 also increased IgM and IgG production induced by suboptimal concentrations of IL-21 by ~2-fold (Fig. 4, *d–f*). In addition, IL-2/IL-10 rendered naive B cells capable of responding to 10-fold less IL-21 (0.1 ng/ml) to produce IgM (Fig. 4*d*). To further explore the interplay between IL-2, IL-10, and

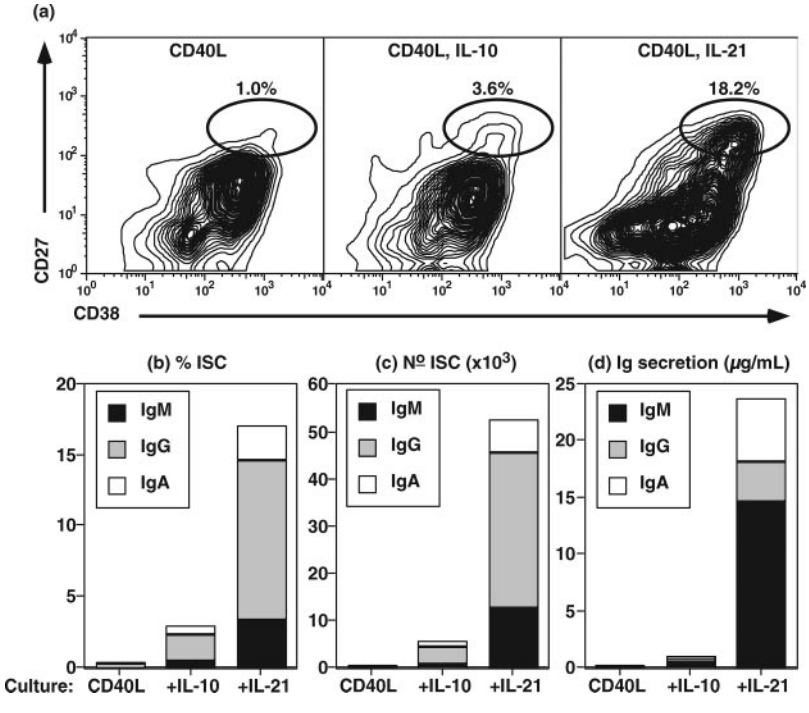

FIGURE 3. GC B cells robustly respond to IL-21. GC B cells were cultured for 4 days with CD40L alone or together with IL-10 or IL-21. *a* and *b*, The frequency of ISC was then determined by phenotype (i.e., $CD38^{high}CD27^{high}$) (*a*) or functional analysis (i.e., ELISPOT) (*b*). *c*, The absolute number of ISC was calculated by multiplying the frequency of ISC (in *b*) by the total number of cells recovered from these cultures. *d*, Ig secretion was determined by ELISA. The values in *a–c* represent the mean of two independent experiments; the values in *d* represent the mean of four independent experiments.

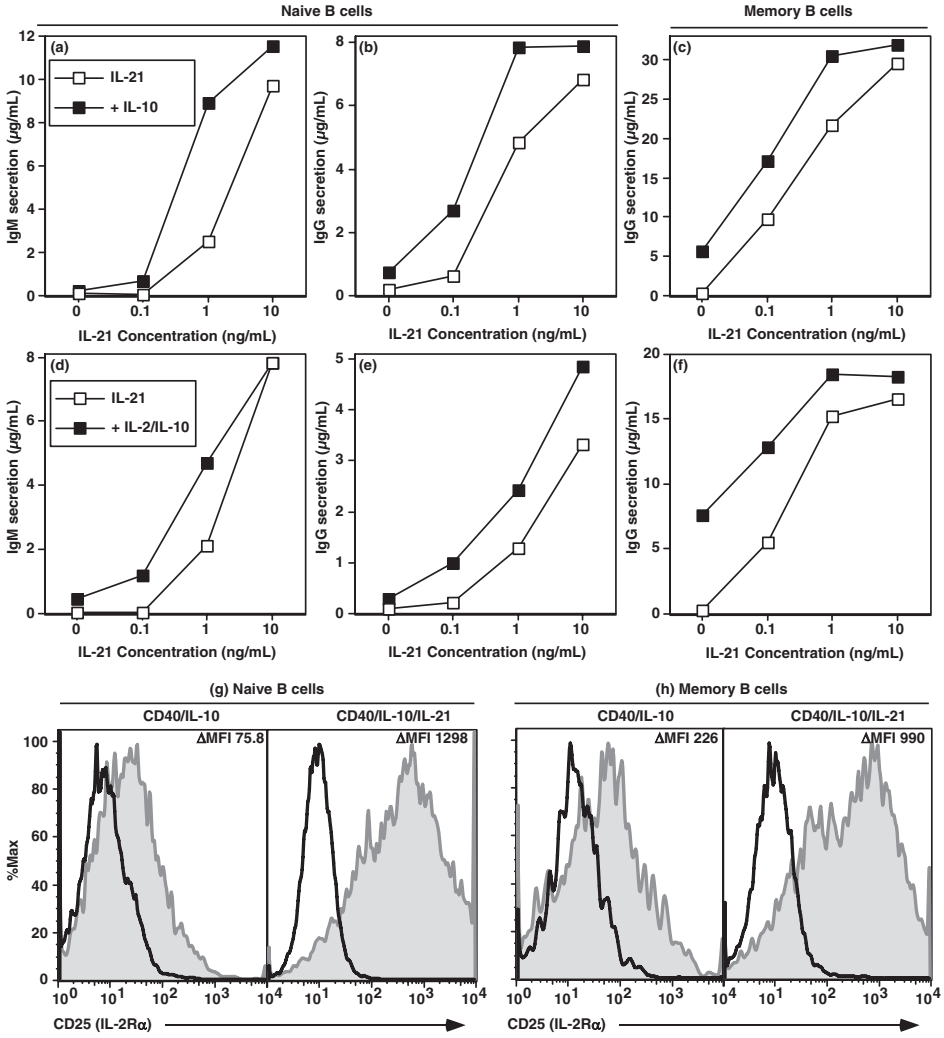

FIGURE 4. IL-10 reduces the dose of IL-21 required to induce Ig secretion by human B cells. *a–f*, Naive (*a*, *b d*, and *e*) and memory (*c* and *f*) B cells were sort purified from human spleens and then cultured with anti-CD40 mAb immobilized on CD32-transfected L cells in the presence of the indicated increasing concentrations of IL-21 (□) alone or together with 100 U/ml IL-10 (*a–c*) or 100 U/ml IL-2 plus IL-10 (■) (*d–f*). Secretion of IgM and IgG was determined after 10 days of culture. Note that the data presented in *a–c* and *d–f* were derived from different experiments using different donor spleens. *g* and *h*, naive (*g*) and memory (*h*) B cells were cultured with anti-CD40 mAb in the absence or presence of IL-10 or IL-21. After 4 days, the cells were harvested and then incubated with an isotype control mAb (black-outlined histogram) or anti-CD25 mAb (solid gray histogram). The values represent the change in mean fluorescence intensity (ΔMFI) calculated as (MFI of CD25) − (MFI of isotype control).

IL-21, expression of the low-affinity IL-2 receptor (IL-2Rα) on activated human B cells was examined. IL-10 up-regulated the expression of IL-2Rα to a greater extent on memory than on naive B cells (Fig. 4, *g* and *h*, *left panels*; Ref. 23). However, IL-21 strongly up-regulated CD25 on both human B cell subsets, such that the level of expression on naive and memory cells was comparable (Fig. 4, *g* and *h*, *right panels*). Thus, IL-21 may modulate the responsiveness of human B cells to the stimulatory effects of IL-2 by regulating the expression of IL-2R.

IL-4 suppresses IL-21-induced ISC differentiation from naive but not memory B cells

IL-4 and IL-21 can act antagonistically, with IL-21 reducing IL-4-induced IgE production by murine B cells (46, 47) and IL-4 suppressing IgG secretion by human B cells stimulated with anti-CD40 mAb and IL-21 (28). However, the ability of IL-4 to inhibit IgG secretion by IL-21-stimulated human B cells was variable, ranging from 0 to 50% (28). We speculated that this variability reflects the fact that total PB B cells—a mix of transitional, naive, and memory B cells that differ in frequency for individual donors—were used in these studies (28) rather than subsets of purified B cells. Furthermore, IL-4 has distinct effects on naive and memory subsets (19, 37) that may not be detected when total B cells are examined.

CD40L alone or CD40L/IL-4 did not induce CB B cells to become ISC, as assessed phenotypically (Fig. 5*a*) or functionally (Fig. 5*b*). Although IL-21 generated functional ISC from CD40L-stimulated CB B cells, this was reduced 2.5–5-fold by IL-4 (Fig. 5, *a* and *b*). When differentiation was assessed in the context of division, IL-4 delayed the generation of ISC from CD40L/IL-21-stimulated B cells by several divisions (Fig. 5*c*). Next, we examined PB B cells, because these cells were used in the original study that reported inhibitory effects, albeit variable, of IL-4 on IL-21-induced Ig secretion (28). PB transitional, naive, and memory B cells secreted large amounts of Ig following stimulation with CD40L/IL-21; however, Ig secretion by memory B cells exceeded that of transitional and naive B cells, especially with respect to IgG and IgA production (Table II). IL-4 had little effect on Ig secretion by CD40L-stimulated PB B cells. However, it reduced IgM, IgG, and IgA secreted by CD40L/IL-21-stimulated transitional and naive cells by ~3- to 5-fold (Table II). In stark contrast, CD40L/IL-21-stimulated memory B cells continued to secrete large amounts of Ig irrespective of the presence of IL-4 (Table II). Similarly, while IL-4 reduced the percentage of ISC generated from CD40L/IL-21-stimulated splenic naive B cells by ~50% (Fig. 5*d*, *left panel*), it had only a small effect on CD40L/IL-21-stimulated splenic memory B cells (32 vs 27%; Fig. 5*e*, *left panel*). When division-linked differentiation of splenic B cell subsets was examined, IL-4 clearly reduced the frequency of ISC generated from CD40L/IL-21-stimulated naive B cells by >2-fold per division (Fig. 5*d*, *right panel*). In contrast, IL-4 did not affect the differentiation rate of CD40L/IL-21-stimulated memory B cells (Fig. 5*e*,

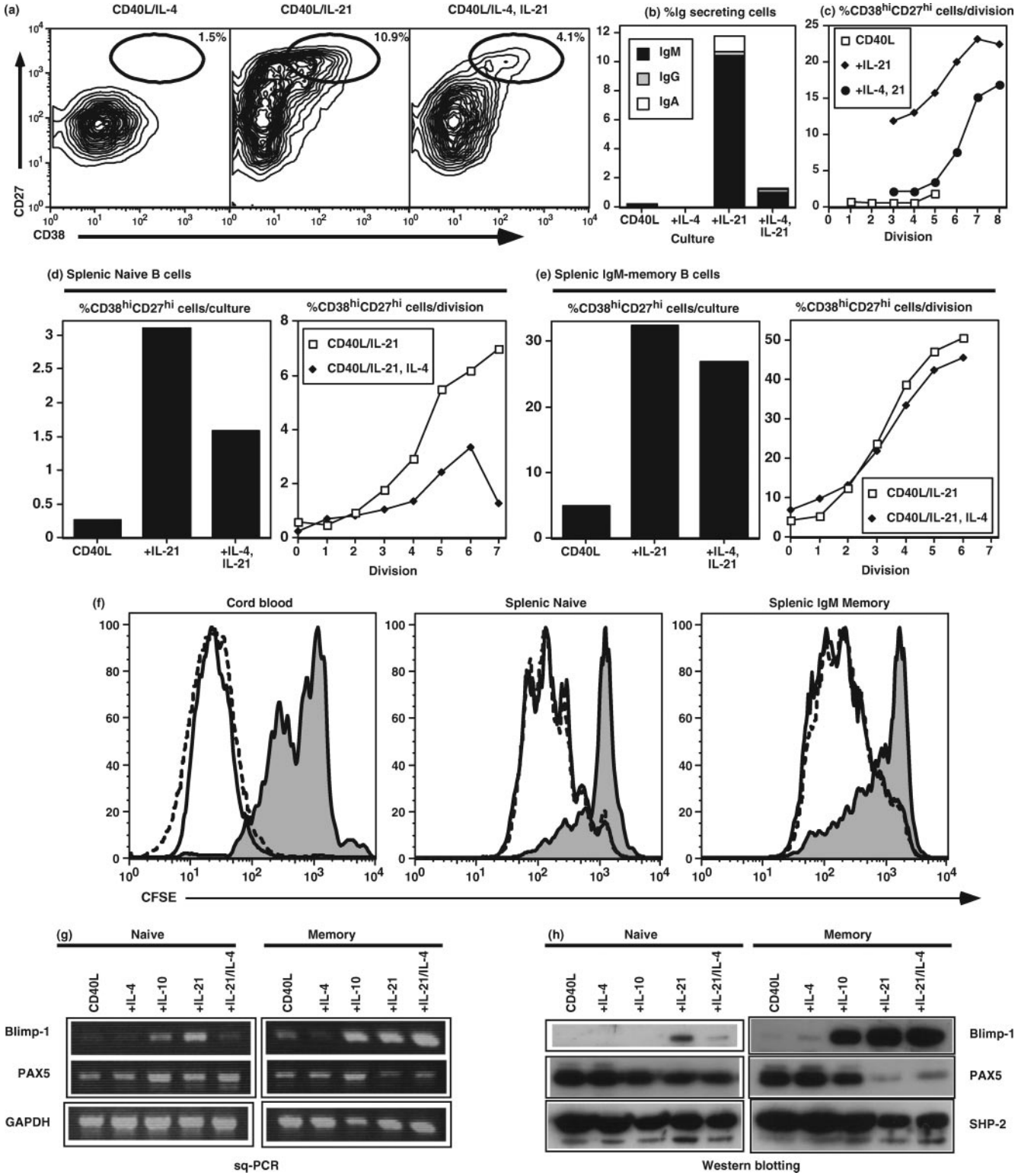

FIGURE 5. IL-4 suppresses IL-21-induced differentiation of transitional and naive but not memory B cells by reducing the expression of Blimp-1. *a–c*, CB B cells were cultured with CD40L and IL-4, CD40L and IL-21, or CD40L, IL-4, and IL-21 for 4 days. The cells were then harvested and the frequency of $CD38^{high}CD27^{high}$ cells (*a*), functional ISC (*b*), and $CD38^{high}CD27^{high}$ cells in each division (*c*) was then determined. The values are the mean of three (*a* and *c*) or two (*b*) independent experiments. *d* and *e*, Naive (*d*) and IgM (*e*) memory splenic B cells were labeled with CFSE and cultured with CD40L alone, CD40L plus IL-21 or CD40L, IL-21, and IL-4. After 4 days, the cells were harvested and the frequency of $CD38^{high}CD27^{high}$ cells per culture as well as in each division was determined. *f*, CB and naive and IgM memory splenic B cells were labeled with CFSE and cultured with CD40L alone (solid gray histogram), CD40L/IL-21 (solid black line), or CD40L/IL-21/IL-4 (dashed black line). After 4 or 5 days, the CFSE profiles of the cells were determined. *g* and *h*, Naive and memory B cells were cultured with CD40L alone or together with IL-4, IL-10, IL-21, or IL-4 and IL-21. After 4 days, RNA was extracted, transcribed into cDNA, and whole cell lysates were prepared. Expression of Blimp-1 and PAX5 was determined by semiquantitative PCR using GAPDH as a standard (*g*) and Western blotting (*h*); SHP-2 expression was assessed to demonstrate similar protein loading.

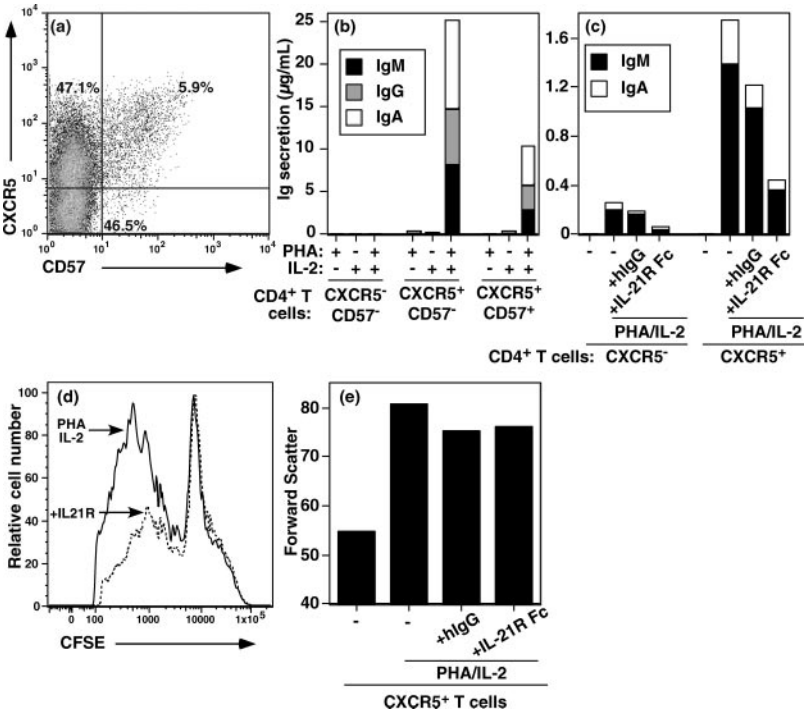

FIGURE 6. Tonsillar T_{FH} cells induce B cell differentiation through an IL-21-dependent mechanism. *a*, Tonsil $CD4^+$ T cells were labeled with mAb to CD57 and CXCR5; the percentage of cells with a $CXCR5^-CD57^-$, $CXCR5^+CD57^-$, or $CXCR5^+CD57^+$ phenotype was determined. *b*, $CXCR5^-CD57^-$, $CXCR5^+CD57^-$, and $CXCR5^+CD57^+CD4^+$ T cells were isolated from human tonsils, treated with mitomycin C, and cocultured with purified autologous B cells in the presence of PHA, IL-2, or PHA and IL-2. Secretion of IgM, IgG, and IgA was determined after 10 days. *c–e*, Tonsil $CXCR5^-$ and $CXCR5^+$ $CD4^+$ T cells were cultured with autologous B cells alone (−) or in the presence of PHA/IL-2 with or without control hIgG1 or IL-21R-Fc. After 5 days, secretion of IgM and IgA (*c*), proliferation of CFSE-labeled B cells (*d*), and T cell size/blastogenesis (*e*) were determined.

right panel). It was possible that IL-4 inhibited IL-21-mediated B cell differentiation by suppressing their proliferation. However, >95% of B cells in cultures stimulated with CD40L/IL-21 with or without IL-4 had undergone more than one division, with most of these cells residing in divisions 3–8 (Fig. 5*f*). Thus, IL-4 suppresses the generation of ISC from CD40L/IL-21-treated naive B cells without affecting cell division. Overall, this analysis of defined B cell subsets from human CB, PB, and spleen clearly revealed the specific inhibitory effect exerted by IL-4 on naive but not memory B cells. The differential sensitivity of transitional/naive and memory B cells to the suppressive effects of IL-4 may explain the variable effect noted by Ettinger and colleagues (28).

IL-4 antagonizes the effects of IL-21 on the differentiation of naive B cells by reducing the expression of Blimp-1

A key mediator of B cell commitment to the PC lineage is Blimp-1 (48). Consistent with this, IL-21 can induce the expression of *Blimp-1* mRNA in activated human and murine B cells (28, 49). Because IL-4 selectively inhibited differentiation of CD40L/IL-21-stimulated naive B cells, we examined the expression of Blimp-1 in B cell subsets activated with CD40L and different cytokines for 4 days at both the mRNA and protein levels. Blimp-1 was not detected in naive B cells activated with CD40L alone or CD40L/IL-4 (Fig. 5, *g* and *h*), yet *Blimp-1* mRNA was detectable following stimulation with CD40L/IL-10 (Fig. 5*g*). In contrast, high levels of Blimp-1 were detected in naive B cells stimulated with CD40L/IL-21 (Fig. 5, *g* and *h*). IL-21-mediated induction of Blimp-1 expression in CD40L-stimulated naive B cells was dramatically reduced by IL-4 (Fig. 5, *g* and *h*), consistent with the ability of IL-4 to suppress the differentiation of naive B cells into ISC.

In contrast to naive cells, Blimp-1 was detected in memory B cells stimulated with CD40L/IL-10 and at greater levels in those cultured with CD40L/IL-21 (Fig. 5*h*). Induction of Blimp-1 in CD40L/IL-21-stimulated memory B cells was accompanied by reduced expression of the transcriptional repressor PAX5 (Fig. 5, *g* and *h*). IL-21-mediated induction of Blimp-1 and loss of PAX5 still occurred in CD40L-stimulated memory B cells in the presence of both IL-4 and IL-21 (Fig. 5, *g* and *h*). Thus, activated memory B cells are insensitive to the modulating effect of IL-4 on IL-21-induced differentiation, which distinctly contrasts naive B cells.

Tonsillar T follicular helper cells induce ISC differentiation of B cells via an IL-21-dependent mechanism

$CXCR5^+$ T_{FH} cells represent ~50% of tonsillar $CD4^+$ T cells (Fig. 6*a*) and provide help to B cells in vitro (32, 44, 50, 51). However, the exact mechanism by which they induce B cell differentiation remains unknown. Because T_{FH} cells express IL-21 (29–32), we assessed the contribution of endogenously derived IL-21 to the ability of T_{FH} cells to activate B cells. An in vitro system was established where tonsil $CD4^+CXCR5^-$ (i.e., non-T_{FH}) and $CD4^+CXCR5^+$ (i.e., T_{FH}) cells were cocultured with autologous B cells and T cell mitogens. The $CD4^+CXCR5^+$ T cell population is comprised of $CD57^-$ and $CD57^+$ subsets (Fig. 6*a*; Ref. 44). Thus, we assessed the functional activity of both $CD57^-$ and $CD57^+$ T_{FH} cells. In the presence of PHA and IL-2, both $CXCR5^+$ T cell subsets induced Ig secretion from cocultured B cells, while $CXCR5^-CD4^+$ T cells had little effect (Fig. 6*b*). For these reasons, we focused on total $CXCR5^+CD4^+$ T cells. Next, we examined the consequences of neutralizing IL-21 with an IL-21R-Fc fusion protein on the ability of activated T_{FH} cells to induce B cell differentiation. Because the Fc portion of the fusion protein was from human IgG, the control for these experiments was human IgG1. Consequently, IgG secretion could not be analyzed in these cultures. The high levels of IgM and IgA (Fig. 6*c*) and number of ISC (not shown) induced by $CXCR5^+$ T cells were reduced 4- to 5-fold by IL-21R-Fc. Although activated $CD4^+CXCR5^-$ T cells induced Ig secretion and ISC at a level equalling ~10% of $CXCR5^+$ T cells, their efficacy was also reduced ~5-fold by IL-21R-Fc (Fig. 6*c*). Because B cell differentiation is division linked, we examined the effect of neutralizing IL-21 on the proliferation of CFSE-labeled B cells. In the presence of PHA/IL-2-activated T_{FH} cells, 68.2% of B cells underwent

more than one division. However, only 48% of B cells were induced to proliferate by T_{FH} cells when endogenous IL-21 was sequestered (Fig. 6*d*).

T_{FH} cells also express IL-21R (32). It was therefore possible that IL-21 acted in an autocrine manner to activate T cells, thereby indirectly mediating B cell differentiation. Therefore, we determined whether the activation status of cocultured T cells was affected by neutralizing IL-21. For this experiment, blastogenesis was used as a readout of T cell activation. When $CD4^+$ T cells were cultured with B cells without PHA/IL-2, they retained the morphological characteristics of resting cells. However, PHA/IL-2 increased cell size (i.e., forward scatter) by ~60%, and this increase was unaffected by IL-21R-Fc (Fig. 6*e*). This suggests that T_{FH} cell-derived IL-21 acts directly on B cells and that the production of IL-21 represents a major mechanism by which T_{FH} cells mediates the differentiation of B cells into ISC.

Discussion

Over the past 5 years, several studies have demonstrated that IL-21 can induce proliferation and differentiation of human and murine B cells stimulated through CD40 (25, 26, 28, 37, 49). The data presented here significantly extend these findings. We found that IL-21 induced the secretion of vast quantities of IgM, IgG, and IgA by all subsets of mature human B cells—transitional, naive, GC, IgM-memory and isotype switched memory—isolated from CB, PB, tonsils, and spleen. Although IL-21 can induce IgM and IgG (26, 28), this is the first description of its ability to induce IgA secretion by human B cells. Notably, IL-21 induced 20- to 1000-fold more Ig secretion by CD40L-stimulated B cells than IL-10 and yielded ISC from naive B cells, a function not exhibited by IL-10. This is a significant finding, because IL-10 has been considered the most potent cytokine for ISC differentiation from human B cells (12). The ability of IL-21 to induce differentiation of naive B cells into ISC suggests that IL-21 may have a major role in primary responses to Ag.

The copious quantities of Ig induced by naive, GC, and memory B cells stimulated with CD40L and IL-21 resulted from their rapid differentiation into ISC. Induction of Blimp-1 correlated with the ability of human B cells to become ISC following stimulation with appropriate cytokines. Thus, IL-21 induced Blimp-1 expression in CD40L-stimulated naive and memory B cells, while IL-10 induced Blimp-1 only in memory cells. The rates of differentiation of CD40L/IL-21-stimulated IgM memory and isotype-switched memory B cells into ISC and the levels of Ig secreted by these cells were comparable. This is consistent with our previous findings that the proliferation rates of these B cell subsets were equivalent and exceeded that of naive B cells (23, 38, 52). Together, these data highlight the functional similarities of IgM memory and switched memory B cells and emphasize that memory cells are not dependent on the acquisition of downstream Ig isotypes for them to respond more rapidly than naive B cells.

When IL-21 was used at saturating doses, IL-2 and/or IL-10 had little effect on CD40-induced B cell differentiation. However, the stimulatory effect of IL-21 was increased by these cytokines when IL-21 was used at suboptimal doses. The net effect was a 10-fold reduction in the amount of IL-21 required to induce Ig secretion and 2-to 5-fold more Ig secreted. This highlights the potential interplay of these cytokines in vivo. IL-21 also potently up-regulated CD25 expression on activated naive and memory human B cells, akin to the ability of IL-10 to induce greater expression of CD25 on memory than on naive B cells (23, 45). In contrast to IL-2 and IL-10, IL-4 potently inhibited the stimulatory effect of IL-21. Strikingly, this inhibitory effect was restricted to naïve—CB and transitional—B cells with only a minor effect on memory cells. The selective effect of IL-4 on naive B cells is consistent with the greater expression of IL-4R on naive B cells (19, 37). IL-4 achieved this inhibitory effect by suppressing the induction of Blimp-1, which is required for commitment to the ISC lineage (48). This may have been achieved by sustaining expression of PAX5, which directly represses Blimp-1 expression (53). The rationale for the inhibitory effect of IL-4 on naive B cells stimulated with CD40L/IL-21 is unclear. It may be a mechanism operative within a GC that facilitates naive B cells to continue undergoing affinity maturation before differentiating into ISC, whereas it would be advantageous to the host for memory B cells, which presumably acquired a high-affinity Ag-specific BCR following the initial immunizing event, to rapidly differentiate into ISC without the need for additional selection events. This would be consistent with the ability of PAX5 and Blimp-1 to induce and suppress, respectively, activation-induced cytidine deaminase, which is required for somatic mutation (48). A feature of memory B cells is their ability to undergo a more rapid and robust response than naive B cells following Ag encounter (1, 2, 23, 24). One mechanism facilitating this is the differential expression of cell cycle regulators resulting in an increased restriction of entry of naive cells into division than memory cells (52). The finding that CD40L/IL-21-stimulated naive and memory B cells exhibit differential sensitivity to the inhibitory effects of IL-4 reveals another mechanism that allows memory B cells to respond with more rapid kinetics to stimulation than naive B cells. By antagonizing the function of IL-4, it may be possible to improve the responses of naive B cells during primary immune reactions in cases of infection or vaccination or even in immunodeficient patients who lack memory B cells (38, 41, 54, 55).

The potency of IL-21 on B cell differentiation suggests it may have clinical application in immunodeficiency or a pathogenic role in autoimmunity and malignancy. First, T_{FH} cells were found to provide help for B cell differentiation by producing IL-21. Interestingly, T_{FH} cell function is impaired in patients with inherited immunodeficiencies (41, 55, 56). These patients are hypogammaglobulinemic, requiring regular Ig replacement therapy. Thus, controlled delivery of IL-21 may restore B cell differentiation in these patients. Second, because IL-21 is the most potent differentiation factor for human B cells and signals through γc, which is mutated in X-SCID (57), it is likely that an inability to signal through the IL-21R/γc complex, rather than other γc-containing B cell tropic cytokine receptors (IL-2R, IL-4R, IL-15R), underlies profound B cell dysfunction characteristic of X-SCID patients. In contrast, IL-21 is increased in several animal models of human autoimmunity (30, 31, 49, 55), and neutralizing IL-21 reduces the severity of disease in these models (58, 59). Together, these findings reveal a potential role for IL-21 in the pathogenesis of murine autoimmune diseases and, by inference, suggest that IL-21 production or function may be dysregulated in human humoral autoimmune diseases. Interestingly, serum levels of IL-21 were recently found to be increased in a cohort of Chinese patients with Sjögren's syndrome (60). It will be important to confirm these findings and extend them to determine whether serum levels of IL-21 are increased in patients with other autoantibody-mediated diseases such as systemic lupus erythematosus and rheumatoid arthritis.

In addition to immunodeficiency and autoimmunity, IL-21-targeted therapies may be useful in treating angioimmunoblastic T cell lymphoma (AITL), a rare $CD4^+$ T cell lymphoma (61, 62). Recent studies that examined malignant AITL cells recognized that they have many features of T_{FH} cells. Specifically, normal T_{FH} and malignant AITL cells express Bcl-6, CXCR5. and CD40L and produce CXCL13 (29, 32, 63–66). Hallmarks of AITL are B cell

activation, hyperplastic B cell follicles, and hypergammaglobulinemia (61, 62, 67). Furthermore, within reactive lymph nodes, malignant T cells closely associate with activated B cell follicles (62, 67). For this reason it has been proposed that constitutive production of CXCL13 and CD40L by malignant T_{FH} cells results in increased recruitment of B cells into follicles, their aberrant activation, and subsequent hypergammaglobulinemia (65, 66). It is possible that CXCL13 produced by malignant T_{FH} cells underlies the recruitment of large numbers of B cells into the reactive lymph nodes of AITL patients. However, given the potent effects of IL-21 on the human B cell differentiation, it is highly likely that production of IL-21 rather than CXCL13 by AITL cells causes the exaggerated B cell activation and hypergammaglobulinemia characteristic of this disease. Thus, disease severity may be reduced by neutralizing IL-21. In conclusion, our study identifies IL-21 as the most potent differentiation factor for human B cells irrespective of stage of development. The potential association of aberrant production of IL-21 and dysfunctional T_{FH} cells with several different human diseases (31, 55) suggests that strategies aimed at increasing or decreasing IL-21 function may have therapeutic benefit for conditions including humoral immunodeficiency, B cell mediated autoimmune conditions, and $CD4^+$ T cell malignancy.

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Disclosures

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