Kinetics of Human B Cell Behavior and Amplification of Proliferative Responses following Stimulation with IL-21[1]

Kim L. Good,*†‡ Vanessa L. Bryant,*†‡ and Stuart G. Tangye[2]*‡

Although recent studies indicated that IL-21 is an important regulator of human B cell activation, detailed comparison of the effects of IL-21 on distinct B cell subsets have not been performed. Our studies revealed that IL-21R is expressed by naive and germinal center B cells, but not memory or plasma cells. IL-21R was increased on naive and memory B cells following in vitro activation. Investigation into the kinetics and magnitude of responses of human B cells to IL-21 revealed that IL-21 potently augmented proliferation of CD40L-stimulated neonatal, splenic naive, and memory and tonsil germinal center B cells. This response exceeded that induced by IL-4, IL-10, and IL-13, cytokines that also induce B cell proliferation. Remarkably, CD40L/IL-21-stimulated naive B cells underwent the same number of divisions as memory cells and exhibited a greater enhancement in their response compared with CD40L alone than memory B cells. Therefore, IL-21 is a powerful growth factor for naive B cells. This may result from the higher expression of IL-21R on naive, compared with memory, B cells. Stimulation of human B cells with CD40L/IL-21 also induced IL-10 production and activation of STAT3. We propose that IL-21 may have therapeutic application in conditions of immunodeficiency where it could expand naive B cells, the predominant B cell subset in such patients. Conversely, because IL-21 is increased in murine models of lupus, dysregulated IL-21 production may contribute to perturbed B cell homeostasis observed in systemic lupus erythematosus. Thus, antagonizing IL-21 may be a novel strategy for treating Ab-mediated autoimmune diseases. ***The Journal of Immunology,*** **2006, 177: 5236–5247.**

Humoral immune responses and memory "recall" responses are critical for controlling infections and maintaining long-term Ab-mediated immunity (1, 2). The primary Ab response is mediated by naive B cells which become activated following the receipt of signals through the BCR, CD40, TLR, and cytokine receptors (3, 4). Activated naive B cells can enter a germinal center (GC),[3] where they undergo class switch recombination and somatic hypermutation, and then differentiate into either high-affinity plasma cells (PC) or memory B cells (3, 5). Memory B cells mediate long-term protective immunity due to their capacity to generate secondary humoral responses, where they dominate over naive B cells by differentiating rapidly into high-affinity PC (6, 7). This is replicated in vitro, where memory B cells respond more rapidly and robustly than naive B cells to T-dependent (TD) and T-independent (TI) stimuli (4, 8–15).

Although various intrinsic mechanisms can regulate B cell responses (16, 17), extrinsic factors such as cytokines also play an important role in determining the outcome of a humoral immune response. Thus, IL-4, IL-10, and IL-13 can modulate the behavior of human B cells. However, not all human B cell subsets respond identically to particular cytokines. For example, IL-4 and IL-13 protect naive B cells from apoptosis (18), induce their proliferation (19, 20), and mediate Ig isotype-switching to IgG4 and IgE (21). In contrast, IL-10 mediates the differentiation of GC and memory, but not naive, B cells into plasmablasts (10–12, 22). These different responses are often attributable to differential expression of cytokine receptors (13, 18, 19). Consequently, cytokines can influence the outcome of immune responses by favoring one B cell subset to predominate over another at different times.

IL-21 is a member of the IL-2/common γ (γ_c) chain family of cytokines (23–25). It is produced predominantly by $CD4^+$ T follicular helper (T_{FH}) cells (26), which provide help for B cell differentiation (27). Members of this cytokine family play important roles in immunity, highlighted by the finding that mutations in components of the γ_c signaling pathway cause severe combined immunodeficiency (25). Furthermore, Th_F cells and IL-21 are increased in murine models of human systemic lupus erythematosus (SLE) (28, 29), thereby providing a possible link between overproduction of IL-21 and autoimmunity. For these reasons, it is necessary to dissect the effects of IL-21 on human B cell populations.

Previous studies have demonstrated a role for IL-21 in regulating proliferation, apoptosis, isotype switching, and differentiation of murine and human B cells (28, 30–33). However, it remains to be determined whether this cytokine preferentially favors the response of one particular subset of B cells, and how the stimulatory activity of IL-21 compares to other well-characterized B cell growth factors. Furthermore, little information is known regarding expression of the IL-21R on human B cells, and the stimuli that modulate its expression. In this study, we report that IL-21 potently synergized with TD stimuli to induce robust proliferation by

*Centenary Institute of Cancer Medicine and Cell Biology, New South Wales, Australia; †Faculty of Medicine, University of Sydney, New South Wales, Australia; and ‡Garvan Institute of Medical Research, New South Wales, Australia

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[2] Address correspondence and reprint requests to Dr. Stuart G. Tangye, Immunology and Inflammation Department, Garvan Institute of Medical Research, 384 Victoria Street, Darlinghurst 2010, New South Wales, Australia. E-mail address: s.tangye@garvan.org.au

[3] Abbreviations used in this paper: GC, germinal center, PC, plasma cell; SLE, systemic lupus erythematosus; TD, T dependent; TI, T independent; γ_c, common γ chain; T_{FH}, T follicular helper; CB, cord blood; ttfd, time-to-first division; MNC, mononuclear cell; SA, streptavidin; sq-PCR, semiquantitative PCR; rh, recombinant human; B-CLL, B-chronic lymphocytic leukemic; MFI, mean fluorescence intensity; PB, peripheral blood.

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human B cells corresponding to distinct stages of differentiation, including neonatal cord blood (CB), naive, GC, IgM memory and isotype-switched memory B cells. IL-21 also induced CD40L-stimulated B cells to secrete increased levels of IL-10. The effect of IL-21 on B cell proliferation and expansion greatly exceeded that of all other cytokines tested. Although IL-21 enhanced proliferation of both naive and memory B cells, the relative enhancement compared with CD40L alone was greatest for naive B cells. This may result from increased expression of IL-21R on both resting and activated naive B cells, relative to memory B cells. However, overall the proliferative response of memory B cells continued to exceed that of naive B cells, as noted previously for responses of these cells to other stimuli (4, 12–14). Our results suggest that IL-21 may have therapeutic application in conditions of immunodeficiency where it could expand naive B cells, the subset that predominates the B cell compartment in these patients (15, 34–36). In contrast, because abnormal levels of IL-21 have been found in murine models of SLE (28, 29), and IL-10 has a pathogenic role in human lupus (37, 38), dysregulated production of IL-21 may contribute to the aberrant B cell behavior observed in SLE (39, 40). Thus, antagonizing the activity of IL-21 may represent a novel strategy for treating Ab-mediated autoimmune diseases. These proposals are supported by the finding that the function and/or numbers of Th_F cells are reduced or enhanced, respectively, in conditions of immunodeficiency (36, 41) and autoimmunity (29, 42).

Materials and Methods

Abs/reagents

The following Abs were used in this study: unconjugated anti-human IL-10 and IL-6 mAb, PE-anti-CD27, anti-IgM, IgD, IgG mAb, and anti-phospho-STAT3 mAb, biotinylated anti-human IL-10, anti-IgM, IgG, IgA mAb, streptavidin (SA) conjugated to PerCP (SA-PerCP) (BD Pharmingen); FITC-conjugated anti-CD20 and allophycocyanin-conjugated anti-CD38 mAb (BD Biosciences); biotinylated anti-IgD and IgE mAbs, and PE-anti-IgA polyclonal Ab (Southern Biotechnology Associates); PE-anti-CD38, IgE mAbs, SA conjugated to Tricolor (Caltag Laboratories); PE-anti-IL-21R (R&D Systems); biotinylated and allophycocyanin-anti-CD27 mAb (eBioscience). CFSE, SA-Alexa 594, and SA-Alexa 647 were from Molecular Probes; 4′,6′-diamidino-2-phenylindole and demecolcine were from Sigma-Aldrich. Recombinant human (rh) IL-4 and IL-10 were provided by Dr. R. de Waal Malefyt (DNAX Research Institute, Palo Alto, CA). IL-10 standard was from BD Pharmingen. rhIL-13, IL-21, and an IL-6 ELISA kit were purchased from PeproTech. Membranes of insect cells infected with baculovirus expressing recombinant human CD40L were prepared by Dr. M. Kehry (Boehringer Ingelheim, Ridgefield, CT) or Dr. G. Shoebridge and N. Hare (Centenary Institute, Sydney, Australia). All culture plates from BD Labware.

Isolation of human B cells

Human spleens were obtained from cadaveric organ donors (Australian Red Cross Blood Service), and tonsils from patients undergoing surgery at Royal Prince Alfred Hospital. CB samples were collected from King George V Hospital for Mothers and Babies. Institutional human ethics review committees approved all studies. Mononuclear cells (MNC) and total B cells were isolated as described previously (12, 13, 43). Purified B cells were fractionated into subsets of naive, IgM memory, and isotype-switched memory cells according to their differential expression of CD27 and Ig isotypes (13, 44, 45). For molecular analyses, naive ($CD20^+CD27^-IgG^-/A^-/E^-$), IgM memory ($CD20^+CD27^+IgG^-/A^-/E^-$), and switched memory B cells ($CD20^+CD27^+IgG^+/A^+/E^+$) were simultaneously identified and isolated following labeling with mAb to CD20, CD27, and IgG/IgA/IgE. For functional experiments, subsets of memory B cells were purified by labeling with anti-CD20 and anti-CD27 mAb together with mAbs to IgM and IgD or IgG, IgA and IgE to identify IgM^+ and switched memory B cells (13). This strategy of negative isolation of memory subsets avoided cross-linking of the BCR. PC were isolated by sorting $CD20^{low}CD38^{high}$ cells from splenic MNC (46, 47). GC B cells were sorted as $CD20^{high}CD38^{high}$ cells from tonsil MNC (15, 22). Cells were sorted on a FACS $Star^+$, Vantage or Aria flow cytometer (BD Immunohistochemistry Systems). Gating strategies excluded doublets. The postsort purity for each population was typically >98%.

Gene expression analysis

RNA was isolated from each B cell population (RNeasy Total RNA Isolation kit; Qiagen). cRNA was synthesized and amplified using biotin-labeled ribonucleotides and T7 RNA polymerase (26, 48, 49). Biotin-labeled cRNA was then hybridized to Human Genome U133 Set GeneChips (Affymetrix), and resulting data analyzed by Affymetrix Microarray Suite software. Heat maps were generated using Spotfire DecisionSite software (49). Gene expression profiles of cell populations were performed in duplicate from different donors.

Analysis of gene expression by PCR

RNA isolated from sort-purified naive, IgM memory, and switched memory B cells was transcribed into cDNA, normalized for expression of GAPDH and semiquantitative PCR (sq-PCR) analysis performed (46). The primers used were: IL-21R, 5′-GTATTCCACTTCATGGCCGAC, 3′-CCATTTCTTGAAGTCTCCGC; IL-4R, 5′-TCCTCCACTGGACACCCATCC, 3′-GGGCCTGGGTAGCTGAATCTTCC; IL-10Ra, 5′-ACATGAAGGTCTTGCAGGAGCC, 3′-ATGCGGAGGGAGGGTTCTAGG (Sigma-Genosys). sq-PCR was performed at least three times for each gene, using cDNA from different donors for each experiment.

Immunofluorescence staining

MNC were preincubated with mouse IgG (100 μg/ml) and then labeled with anti-CD20, CD27, and a combination of Abs to IgG/IgA/IgE to resolve naive, IgM memory, and switched memory B cells. The cells were also labeled with anti-IL-21R or control mAb and then fixed in 1% formaldehyde. Samples were acquired on a FACSCalibur (BD Immunohistochemistry Systems) and analyzed using FlowJo (Tree Star). To assess expression of phosphorylated STAT3, purified splenic B cells were cultured in the absence or presence of IL-21 in RPMI 1640 medium for 5–15 min. The cells were then harvested, fixed in 2% formaldehyde (37°C, 20 min) and permeabilized with ice-cold methanol (90%) on ice for 30 min. The cells were then incubated with isotype control mAb or anti-phospho-STAT3 mAb (30 min, room temperature) and analyzed as above.

Expression of IL-21R after activation

Sort-purified naive and memory B cells were cultured (8×10^5/ml) in 48-well plates for 3 days in the absence or presence of CD40L. At different times, the cells were harvested and stained with anti-IL-21R mAb. B cells were cultured in Opti-MEM low-serum medium (Invitrogen Life Technologies) containing 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 μg/ml penicillin (Invitrogen Life Technologies), 100 μg/ml streptomycin (Sigma-Aldrich), 40 μg/ml human apotransferrin (Sigma-Aldrich), or bovine apotransferrin (Invitrogen Life Technologies) and 20 μg/ml Normocin (InvivoGen).

B cell proliferation and time to first division assay

Sort-purified B cell subsets ($20–50 \times 10^3$ cells/well/200 μl) were cultured in B cell medium (13) containing Normocin in 96-well round-bottom plates with CD40L alone, or together with IL-4 (400 U/ml), IL-10 (100 U/ml), IL-13 (10 ng/ml), IL-21 (50 ng/ml), or anti-Ig (10 μg/ml). GC B cells ($10–20 \times 10^3$ cells/well/200 μl) were cultured with CD40L alone or with IL-4, IL-10, IL-21, or IL-10 plus IL-21. To determine time-to-first division (ttfd), the mitotic inhibitor demecolcine (10 ng/ml), which allows cells to enter their initial S-phase but blocks all subsequent rounds of division, was added to the cultures (13, 50). [^{3}H]Thymidine incorporation (1 μCi/ml per well; ICN Biomedicals) was measured during a 4-h pulse every 24 h, over a 6-day time period for the ttfd assays, and for 8–18 h for proliferation assays in the absence of demecolcine. Cells were harvested and log normal distributions fitted to the data for ttfd using Prism software (GraphPad Software) (13).

Cell division analysis and cell number determination

The division history of cultured cells was determined by assessing dilution of the fluorescent division tracking dye CFSE. Purified B cell subsets were labeled with CFSE (12, 13) and cultured with CD40L alone, or with IL-4, IL-10, IL-13, or IL-21. After 5 days, the cells were harvested and the percentage of cells in each division was determined by division slicing (12). The absolute number of cells was determined by the addition of a known number of CaliBRITE beads (BD Biosciences) to each well before harvesting the cells (12).

Cytokine ELISAs

Sort-purified B cells ($4-8 \times 10^5$/ml) were cultured in either 48- or 96-well plates with CD40L alone, or in combination with IL-21 or anti-Ig. Supernatants were harvested after 4 days and the amount of IL-6 and IL-10 produced measured by cytokine-specific ELISAs (47, 51). The sensitivities of the ELISAs were 5 pg/ml (IL-10) and 1 ng/ml (IL-6). Absorbances were measured on a Titertek Multiskan MCC/340 ELISA reader (Thermo Labsystems). Analysis was performed using SOFTmax 881 software (Molecular Devices).

Results

Cytokine receptor expression on human mature splenic B cell subsets

Global gene expression was used to assess expression of cytokine receptors on human splenic naive, IgM (nonswitched) memory, isotype-switched memory B cells, and PC. The majority of cytokine receptors examined were not detected in any B cell subset (Fig. 1*a*). However, receptors for cytokines known to stimulate human B cells, such as IL-2, IL-10, and IL-13 (11, 21, 52), displayed relatively uniform expression on resting naive and memory B cells, with reduced expression on PC. IL-6R, IL-15R, and IL-22R were present only on PC, consistent with the responsiveness of PCs to IL-6, but not IL-4 (46). IL-15R has been reported to be expressed by myeloma cells, and IL-15 can act as an autocrine survival factor for these cells (53). However, the functional significance of expression of IL-15R and IL-22R on normal PC is unknown and is currently under investigation. Another striking difference between subsets was the selective expression of IL-4R and IL-21R on naive B cells, whereas both memory B cell subsets and PC lacked these receptors (Fig. 1*a*). Differential expression of cytokine receptors among B cell subsets was confirmed by sq-PCR analysis (IL-4R, IL-10Ra, IL-21R, Fig. 1*b*; IL-13R, data not shown) and flow cytometry (IL-21R, Fig. 1*c*; IL-4R, IL-10R, data not shown). sq-PCR confirmed that naive B cells displayed increased levels of IL-4R and IL-21R compared with either memory B cell subset, while IL-10Ra was expressed comparably by all three subsets (Fig. 1*b*). Furthermore, flow cytometric analysis demonstrated that IL-21R was weakly expressed on naive B cells, yet was absent from memory B cells (Fig. 1*c*).

Expression of IL-21R increases following activation

Although IL-21R was differentially expressed on human resting naive and memory B cells, it was important to establish whether its expression could be modulated following in vitro activation. Indeed, murine B cells and human B-chronic lymphocytic leukemic (B-CLL) cells up-regulate expression of IL-21R following exposure to LPS (33) and CD40L (54), respectively. Thus, expression of IL-21R was assessed following stimulation with CD40L. IL-21R increased on naive and memory B cells in response to culture with CD40L compared with cells cultured without exogenous stimuli (Fig. 2*a*). When the levels of IL-21R were examined over a 72-h time course, its expression was up-regulated more rapidly on CD40L-treated memory B cells compared with naive B cells during the first 12–24 h of culture (Fig. 2*b*). Thereafter, however, the level of IL-21R on naive B cells rose to a greater extent than on memory B cells such that the change in mean fluorescence intensity (MFI) of expression for naive B cells was more than double that for memory B cells. These results suggest that the ability of IL-21 to induce activation and subsequent proliferation of human B cells may differ according to their stage of differentiation.

IL-21 increases the recovery of CD40L-stimulated naive and memory B cells

To determine whether the increased expression of IL-21R on human naive B cells preferentially enhanced their response to in vitro stimulation, relative to memory B cells, the effects of IL-21 on B cell expansion were investigated. The effect of IL-21 was initially compared with that of IL-10, because IL-10 has been well-established as a potent growth factor for human B cells (52). When naive and memory B cells were stimulated with CD40L alone, in general, a greater number of memory B cells were recovered after 5 or 6 days of culture (Table I). In the presence of IL-10, ~2- to 3-fold more cells of each subset were recovered for both times examined compared with cultures treated with CD40L alone (Table I). However, IL-21 induced a much greater expansion of CD40L-stimulated B cells (Table I). After 5 days of stimulation, nearly 10 times more B cells were present in cultures of naive, IgM

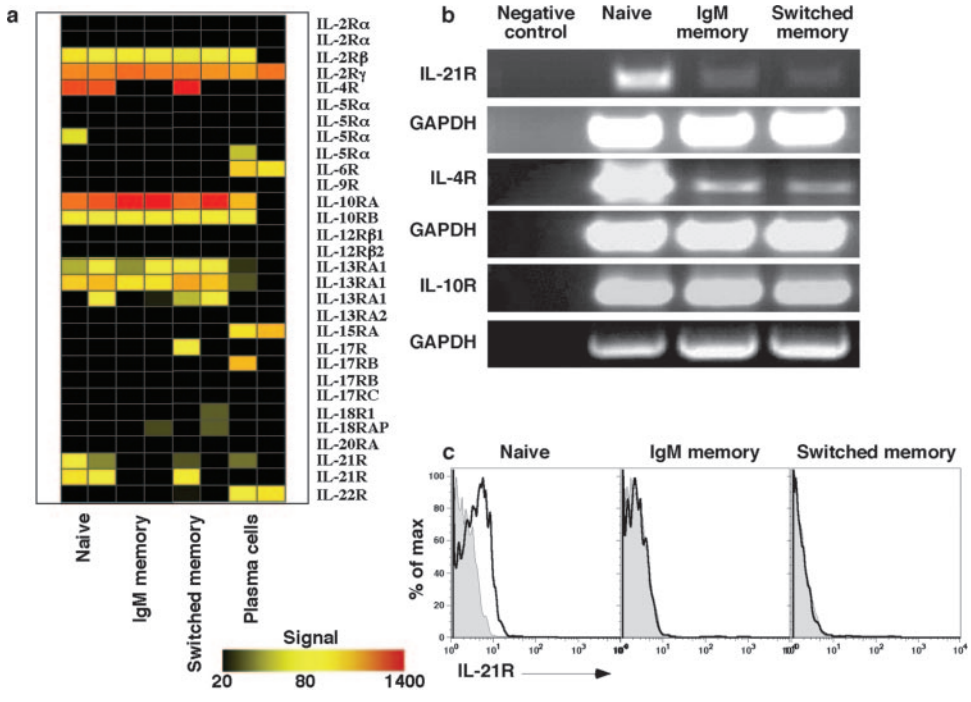

FIGURE 1. Cytokine receptor expression by human splenic B cell subsets. *a*, Microarray gene expression profiles for sort-purified naive, IgM memory, switched memory B cells, and PC. The range of the signal intensity is from 20 to 1400, represented by color shading from black to bright yellow. Each column represents an individual donor, or in the case of PC, different groups of pooled donors. *b*, Gene expression analysis was confirmed by sq-PCR, using GAPDH as a standard. The gel is representative of three PCRs using cDNA prepared from different donors. *c*, Splenic B cells were stained with mAbs to CD20, CD27, and Ig isotypes to resolve B cell populations, together with anti-IL-21R mAb (bold line histogram) or an isotype control (shaded histogram).

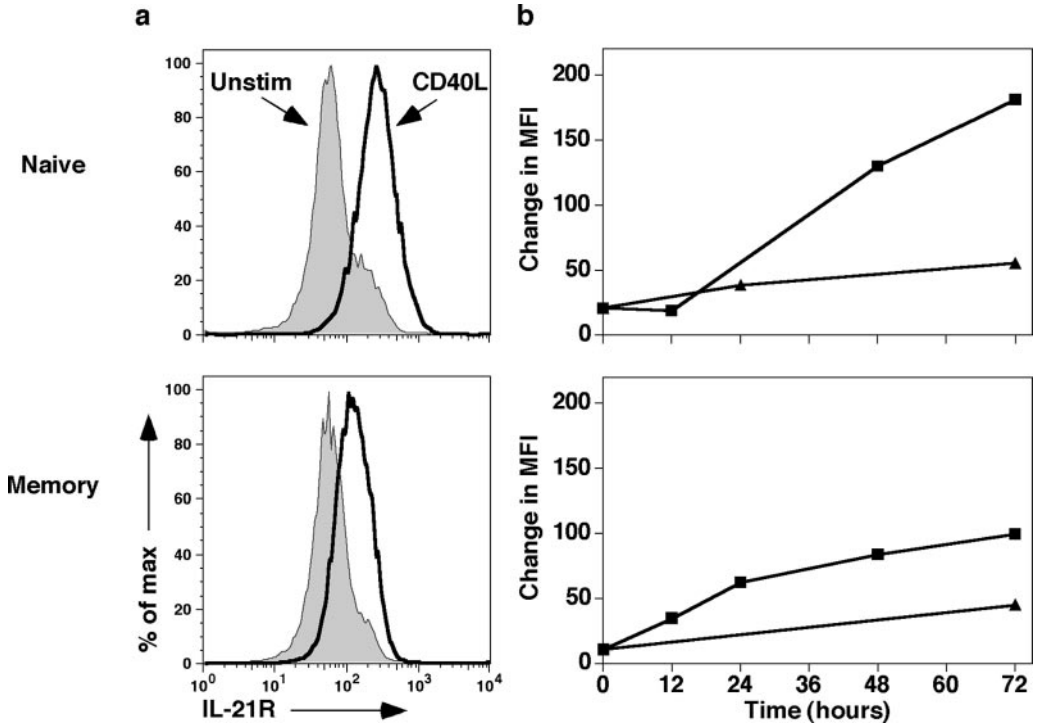

FIGURE 2. IL-21R expression on activated naive and memory B cells. Sort-purified naive and memory B cells were cultured in the absence (shaded histogram/"Unstim" in *a*; △ in *b*) or presence of CD40L (bold histogram/"CD40L" in *a*; ■ in *b*). Cells were harvested after (*a*) 72 h or (*b*) different times over 3 days and a stained with anti-IL-21R mAb or isotype control. The data are presented as the change in MFI (MFI (receptor mAb) − MFI (control mAb)) for each time point and culture condition, and are representative of three experiments.

memory, and switched memory B cells stimulated with CD40L and IL-21 compared with those stimulated with only CD40L (Table I). The potent effect of IL-21 on the proliferation of naive B cells was also evident after 6 days of culture; however, by this time the numbers of surviving IgM and switched memory B cells that had been stimulated with CD40L and IL-21 declined, most likely due to the exhaustion of the culture and subsequent death due to growth factor deprivation. Despite this, these initial studies established that IL-21 was capable of inducing vigorous proliferation of naive and memory B cell subsets in vitro. These findings were examined in further detail by investigating cell division using CFSE.

Human B cells undergo further rounds of division in response to IL-21 compared with other cytokines

When stimulated with CD40L alone, most naive B cells remained undivided, with only a small proportion of cells appearing in the first few divisions. In contrast, most IgM memory and switched memory B cells underwent at least two rounds of division in response to CD40L (Fig. 3*a*). Addition of various cytokines to cultures of CD40L-stimulated B cells modulated their proliferative behavior. IL-4 and IL-13 had an almost identical effect on activated naive B cells, increasing the percentage of cells in the first and second divisions. These cytokines had a similar, but lesser, effect on IgM memory B cells, and essentially no effect on the response of switched memory B cells (Fig. 3*a*). In contrast, naive and memory B cells that were stimulated with CD40L and IL-10 underwent further divisions than those cultured with CD40L alone, with cells distributed evenly across divisions two to four and a concurrent reduction in the percentage of undivided cells. Although IL-10 markedly enhanced proliferation, IL-21 induced a greater increase in proliferation of all B cell subsets. In the presence of CD40L and IL-21, >90% of harvested cells had undergone one or more cell divisions, with the majority of IL-21-stimulated

Table I. *Comparison of the effects of IL-10 and IL-21 on the recovery of naive and memory B cells activated with CD40L*[a]

	Cell Number ($\times 10^3$)					
	Day 5			Day 6		
Stimulus	Naive	IgM memory	Switched memory	Naive	IgM memory	Switched memory
CD40L	74.3 ± 52 ($n = 4$)	67.9 ± 27.3 ($n = 4$)	117.6 ± 17.3 ($n = 2$)	153.9 ± 96.0 ($n = 5$)	362.0 ± 198.3 ($n = 5$)	366.5 ± 134 ($n = 5$)
CD40L + IL-10	142.7 ± 82.7 ($n = 3$)	197.4 ± 90.8 ($n = 3$)	310.7 ± 67.7 ($n = 2$)	399.5 ± 153 ($n = 5$)	467.1 ± 123 ($n = 5$)	740.5 ± 213 ($n = 5$)
CD40L + IL-21	623.4 ± 390.6 ($n = 4$)	588.2 ± 246 ($n = 4$)	852 ± 357 ($n = 2$)	1470.9 ± 312 ($n = 5$)	473 ± 98.4 ($n = 5$)	398 ± 117.3 ($n = 5$)

[a] Sort-purified naive, IgM memory, and switched memory B cells (200×10^3 cells/well) were cultured with CD40L alone or in combination with IL-10 or IL-21. A known number of CaliBRITE beads were added to each well before harvesting to enumerate cell populations after either 5 or 6 days of culture. Each value represents the mean ± SD of the indicated number of experiments performed using cells isolated from different donor spleens.

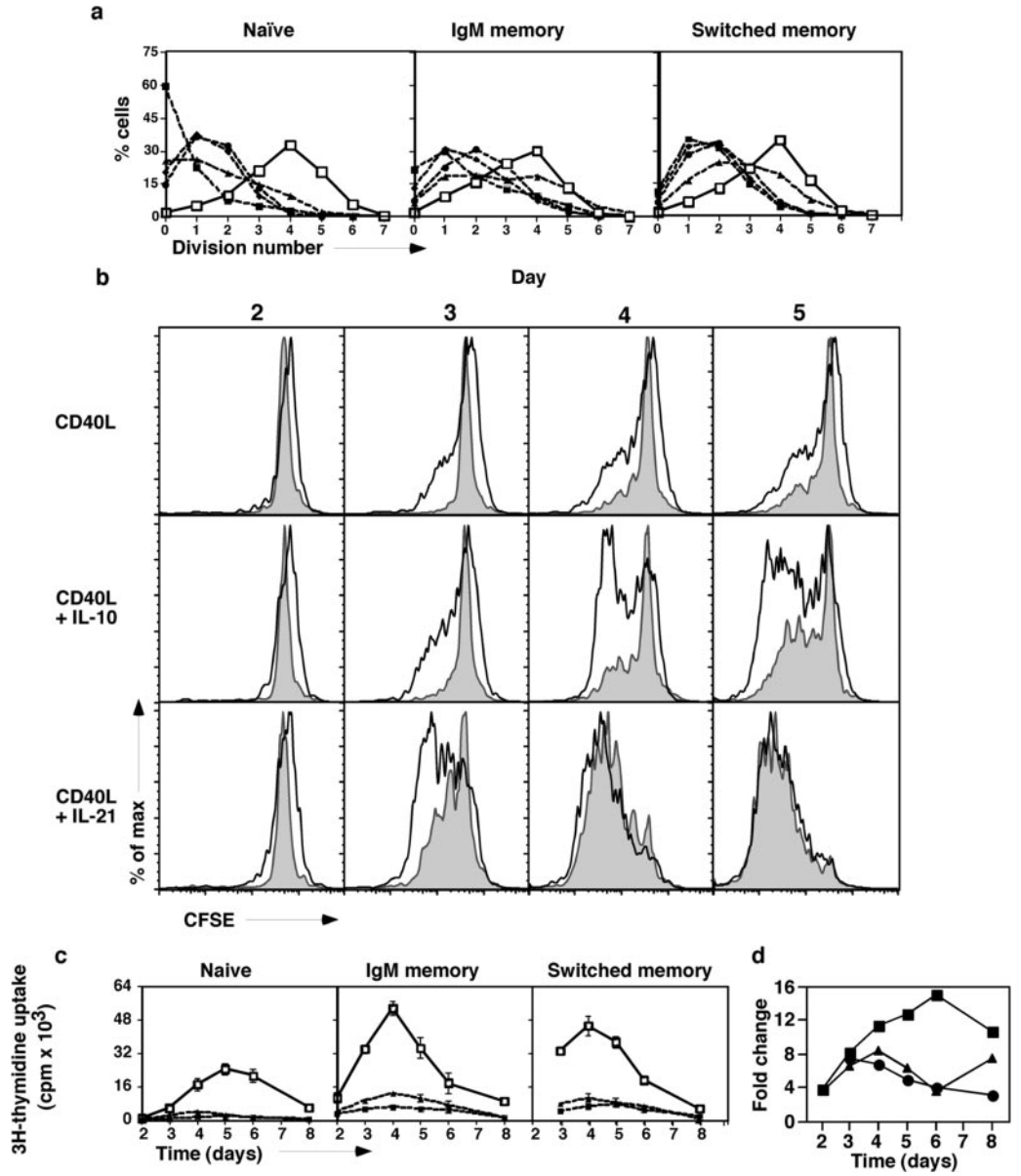

FIGURE 3. IL-21 induces CD40L-stimulated human B cells to undergo the greatest number of divisions compared with other cytokines. *a*, Sort-purified B cells were labeled with CFSE and cultured with CD40L alone (■) or in combination with IL-4 (●; 400 U/ml), IL-10 (▲; 100 U/ml), IL-13 (♦; 10 ng/ml), or IL-21 (□; 50 ng/ml) for 6 days. The percentage of cells per division was assessed by division slicing. *b*, Sort-purified CFSE-labeled naive B cells (shaded histogram) and IgM memory B cells (bold line histogram) were cultured with CD40L alone or in combination with IL-10 or IL-21 and harvested after 2, 3, 4, and 5 days. *c*, Sort-purified B cells were cultured with CD40L alone (■) or together with IL-10 (▲) or IL-21 (□). Proliferation was assessed every 24 h by pulsing the cultures with [^{3}H]thymidine and harvesting 8 h later. Each point represents the mean ± SEM of triplicate samples. *d*, Fold change of [^{3}H]thymidine incorporation by CD40L/IL-21-stimulated naive (■), IgM memory (▲), and switched memory (●) B cells over corresponding CD40L alone-stimulated cells. This data are representative of at least three experiments using cells from different donors.

cells being detected in later divisions (Fig. 3*a*). Addition of IL-4, IL-10, or IL-13 to cultures of B cells stimulated with CD40L and IL-21 did not synergize with IL-21 to result in a greater proliferative response (data not shown). Surprisingly, CFSE division profiles of naive and both memory B cell subsets stimulated with CD40L and IL-21 for 6 days were very similar. This was unexpected because memory B cells have been found to proliferate more than naive B cells when stimulated with CD40L alone or in combination with IL-4, IL-10, IL-13 (Fig. 3*a*; Refs. 12, 13, 15), NKT cells (14), or TI stimuli (4, 15).

Kinetics of the proliferative response of naive and memory B cells to IL-21 was investigated by comparing CFSE profiles of activated naive and IgM memory B cells after 2, 3, 4, and 5 days of culture. Irrespective of the time of harvest, when memory B cells were stimulated with CD40L alone or in combination with IL-10, they underwent more rounds of division than naive B cells (Fig. 3*b*, *upper* and *middle panels*). After culture with CD40L and IL-21 for 3 days, IgM memory B cells had also proliferated further than naive B cells (Fig. 3*b*, *lower panel*). However, on both days 4 and 5, the CFSE profiles of naive and IgM memory B cells were superimposable, indicating that naive B cells had proliferated to the same extent as memory B cells, a phenomenon not observed in any other cultures (Fig. 3*b*; Refs. 4, 12–15). Similar findings were obtained when the kinetics of B cell proliferation were studied using a [^{3}H]thymidine-based assay. Here, CD40L induced a low but detectable level of proliferation in all B cell subsets at most

times examined, with the peak response occurring between days 3 and 5 (Fig. 3*c*). Consistent with the CFSE data, uptake of [^{3}H]thymidine by memory B cells exceeded that by naive B cells for all time points. IL-10 increased [^{3}H]thymidine incorporation by naive and memory B cells ~2- to 3-fold. However, IL-21 had a much greater effect on each B cell subset (Fig. 3*c*). Although the magnitude of the proliferative response following stimulation with CD40L and IL-21 was greatest for memory B cells, the fold-increase in [^{3}H]thymidine incorporation relative to that induced by CD40L alone was greatest for naive B cells (Fig. 3*d*). Uptake of [^{3}H]thymidine by CD40L-stimulated naive B cells was increased 10- to 15-fold by IL-21, while the relative increase of IgM memory and switched memory B cells was only 4- to 8-fold. The response of memory cells was maximal following 4 days of stimulation with CD40L and IL-21 and rapidly declined thereafter (Fig. 3, *c* and *d*). In contrast, maximal proliferation of CD40L/IL-21-stimulated naive B cells occurred on day 5 and was maintained at this level for at least another 24 h (Fig. 3, *c* and *d*). Thus, the sustained maximal response of naive B cells stimulated with CD40L and IL-21 (Fig. 3*c*), coupled with their greater stimulation index (Fig. 3*d*) and concurrent exhaustion of memory B cells cultures, may facilitate naive B cells to undergo similar rounds of division as memory B cells, as revealed by dilution of CFSE (Fig. 3, *a* and *b*).

IL-21 reduces the ttfd of naive and memory B cells

The effect of IL-21 on different parameters of B cell proliferation was next investigated by determining the time required for B cells to enter their first division, as well as the relative frequency of B cells recruited into division in response to stimulation with CD40L in the absence or presence of IL-21 or other stimulatory cytokines. This was achieved by assessing proliferation in the presence of the mitotic inhibitory drug demecolcine, which allows the cells to enter their initial S-phase but prevents all subsequent rounds of cell division (13, 50). CD40L-stimulated IgM memory and switched memory B cells entered their first division substantially earlier than naive B cells (Fig. 4, *a* and *b*), consistent with our previous studies (13). By comparing the amplitude of the fitted curves, it was also apparent that ~2- to 3-fold more memory B cells were recruited into division than naive B cells (Fig. 4, *a* and *c*). Inclusion of IL-4, IL-10, IL-13, or IL-21 in cultures of CD40L-stimulated B cells had differential effects on the ttfd. IL-4 and IL-13 did not notably change the proliferation characteristics of the B cell subsets, while IL-10 reduced the ttfd of both naive and memory B cells by 5–6 h, and increased the amplitude of the response (i.e., frequency of cells recruited) by 1.5- to 2-fold (Fig. 4*a*; Ref. 13). In contrast to the other cytokines examined, IL-21 reduced the ttfd of CD40L-stimulated B cells by ~20% (14.3 h for naive; 11.4 h for IgM memory and switched memory; Fig. 4, *a* and *b*). Consequently, the ttfd of B cells cultured with CD40L and IL-21 approximated that of cells cultured with CD40L and anti-Ig (Fig. 4, *a* and *b*), a TD stimulus that in our hands induces the highest level of B cell proliferation in vitro. Thus, although IL-21R was expressed only by resting naive B cells, the up-regulation in its expression on both naive and memory B cells allows IL-21 to substantially modulate the proliferative responses of both subsets when costimulated with CD40L.

The effect of IL-21 on recruitment of cells into division was quantitated by comparing the amplitudes of the fitted curves from different experiments (Fig. 4, *a* and *c*). Although IL-21 or anti-Ig reduced the ttfd of CD40L-stimulated naive and memory B cells to a similar degree (i.e., ~20%), they differed in their ability to influence the frequency of responding memory B cells. The frequency of naive B cells recruited into division following stimulation with CD40L was increased ~5-fold by IL-21 or anti-Ig. In contrast, the effect of IL-21 on recruitment of memory B cell subsets into division was less than that of anti-Ig (i.e., 2- to 3-fold increase in amplitude with IL-21 compared with 5-fold increase with anti-Ig; Fig. 4*c*). If the combination of CD40L and anti-Ig is

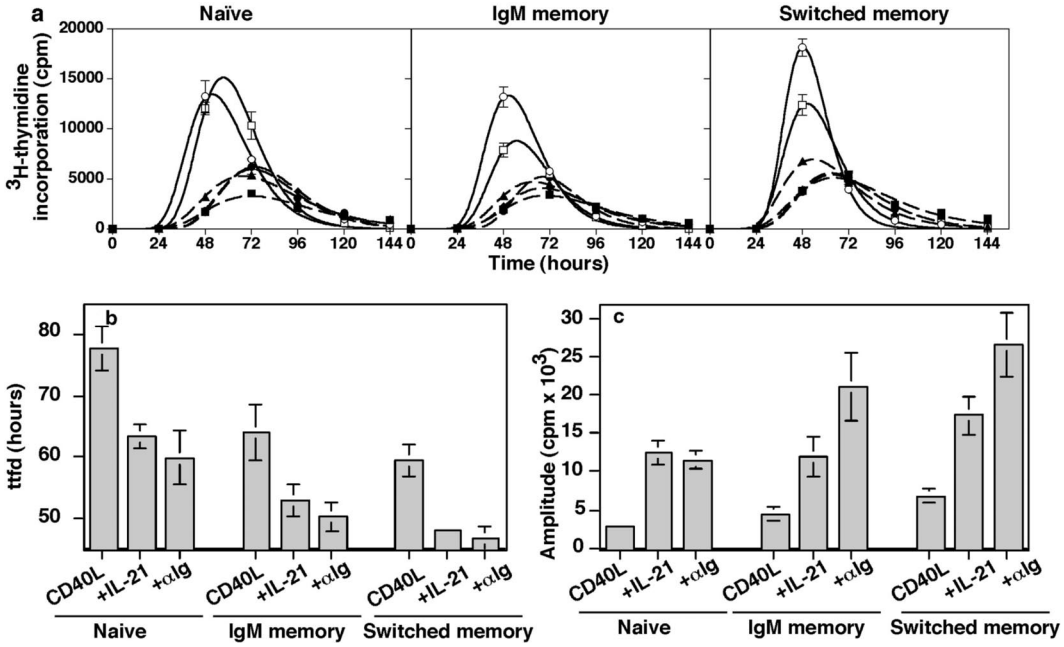

FIGURE 4. Naive B cells are maximally stimulated by IL-21 to enter division. *a*, Sort-purified B cells were cultured with CD40L alone (■) or in combination with IL-4 (●), IL-10 (▲), IL-13 (♦), IL-21 (□), or anti-Ig (○). Proliferation was assessed every 24 h by the incorporation of [^{3}H]thymidine during a 4-h pulse. The mitotic inhibitor demecolcine was added at the onset of cultures to measure entry of the cells into their first S-phase, and therefore their ttfd. The center of the curve is taken as the mean ttfd, while the height is proportional to the number of cells entering division. The mean ttfd (*b*) and amplitude (*c*) of naive and memory B cell subsets stimulated with CD40L alone or in combination with IL-21 or anti-Ig from multiple experiments was determined. The graphs represent the mean ± SEM of three to six independent experiments.

assumed to lead to the largest number of B cells entering their first division, this result would imply that IL-21 can induce the same maximal response in naive B cells whereas its effect on memory B cells is ~50% of the maximum. The basal level of expression of IL-21R on naive cells, but not memory cells, coupled with increased expression on activated naive vs memory B cells, may contribute to the ability of IL-21 to induce a more potent change in the proliferative capability of naive B cells relative to memory B cells.

IL-21 enhances the proliferation of neonatal B cells

From the data already presented, it was apparent that IL-21 synergized with signals delivered by CD40L to induce a potent proliferative response from naive and memory B cells. Recently, Ettinger et al. (30) described the ability of IL-21 to induce proliferation of naive B cells present in CB. We extended these findings by assessing expression of IL-21R on CB B cells, and comparing their response to IL-21 to that induced by other cytokines. CB B cells also expressed detectable levels of IL-21R (Fig. 5*a*). Next, CB B cells were cultured with CD40L alone or in the presence of IL-21 or other γ_c chain-binding cytokines (IL-2, IL-4, IL-15), or those known to enhance B cell proliferation (IL-10, IL-13) for 4 days. In the presence of CD40L alone, CB B cells underwent proliferation, evidenced by incorporation of [^{3}H]thymidine (Fig. 5*b*). IL-2, IL-10, or IL-15 had only a mild effect on this response, enhancing proliferation by 1.3-, 1.9-, and 1.4-fold, respectively, while IL-4 and IL-13 had a much greater effect (3.5- to 5-fold increase; Fig. 5*b*; mean of four experiments). However, IL-21 had the greatest effect, increasing uptake of [^{3}H]thymidine by CB B cells by ~8.0-fold over that observed for CD40L alone (Fig. 5*b*; $n = 4$). When IL-21 was combined with IL-4 or IL-10, there was no further increase in proliferation of CD40L-stimulated CB B cells (Fig. 5*c*). This contrasts the ability of IL-4 and IL-10 to have an additive effect on proliferation of human B cells stimulated through the BCR or CD40 (52), but is consistent with the lack of synergy between IL-21 and either IL-4, IL-10, or IL-13 on CD40L-stimulated human naive and memory B cells (data not shown).

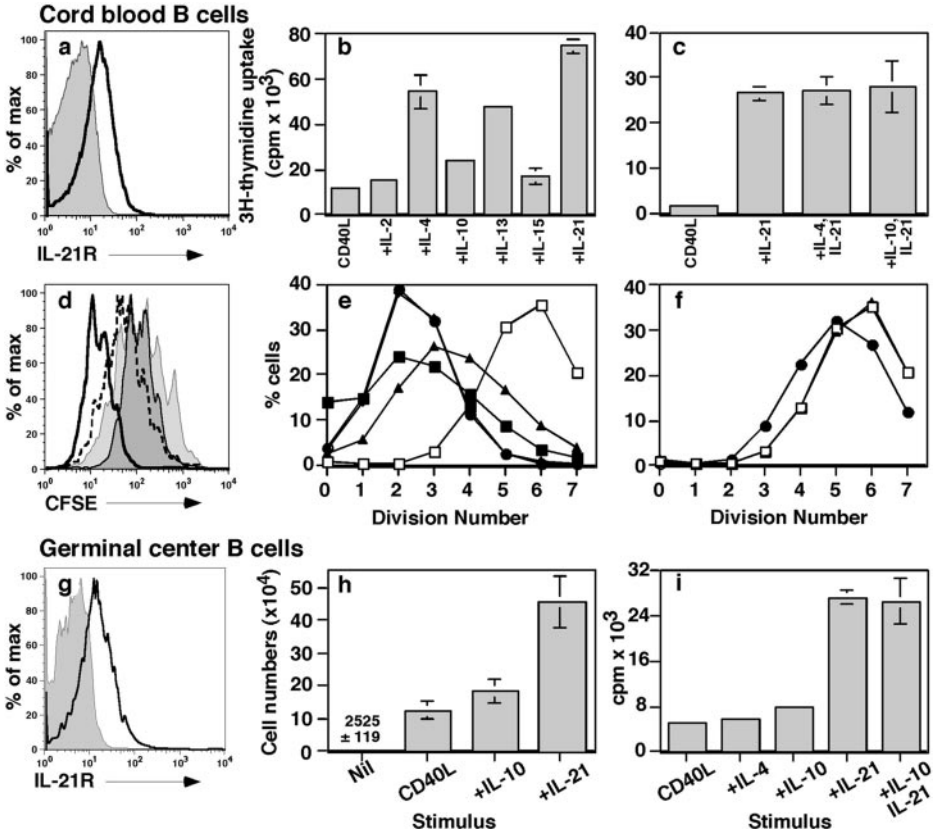

FIGURE 5. IL-21 potently stimulates proliferation of both CB and GC B cells. *a*, Expression of IL-21R on CB B cells; the overlay and solid histograms represent the fluorescence of cells incubated with anti-IL-21R and a control mAb, respectively. *b* and *c*, Purified CB B cells were cultured with CD40L alone, or in the presence of (*b*) IL-2, IL-4, IL-10, IL-13, IL-15, IL-21, or (*c*) IL-21, IL-4 plus IL-21, or IL-10 plus IL-21. Proliferation was measured by determining the incorporation of [^{3}H]thymidine into newly synthesized DNA during the final 18 h of a 4-day culture. Note, the data in *b* and *c* are from different experiments. These results are representative of four independent experiments performed using cells from different donors. *d–f*, CFSE-labeled CB B cells were cultured with either (*d* and *e*) CD40L alone (light gray solid histogram in *d*, ■ in *e*), or in the presence of IL-4 (dark gray solid histogram in *d*, ◇ in *e*), IL-10 (dotted overlay in *d*, ● in *e*), IL-13 (♦ in *e*), or IL-21 (bold overlay in *d*, □ in *e*), or *f* with CD40L and IL-21 (□), or in combination with IL-4 (○) or IL-10 (▲). The CFSE profiles (*d*), and the percentage of cells present in each division (*e* and *f*) were determined after 6 days of culture. Note that the responses to IL-4 and IL-13 in *e* are identical; thus, individual symbols cannot be resolved. *g*, Tonsil GC B cells were stained with either anti-IL-21R mAb (bold line histogram) or an isotype control (shaded histogram). *h*, Sort-purified (CD38^{high}CD20^{high}) GC B cells (~2 × 10^5/well/400 μl) were either unstimulated or cultured with CD40L alone or together with IL-10 (100 U/ml) or IL-21 (50 ng/ml). After 4 days, the absolute number of surviving cells was determined. The values represent the mean ± SEM of four independent experiments using B cells isolated from different tonsils. *i*, GC B cells (~10 × 10^3/well/100 μl) were cultured with CD40L alone, or in the presence of IL-4, IL-10, IL-21, or IL-10 plus IL-21. Proliferation was measured by determining incorporation of [^{3}H]thymidine into newly synthesized DNA during the final 18 h of a 4-day culture. The values represent the mean ± SD of triplicate cultures.

The effect of IL-21 on proliferation of CB B cells was also examined by assessing their division history by labeling them with CFSE. Following stimulation with CD40L for 6 days, ~15% of CB B cells remained undivided, while the remaining cells could be found in divisions one through six; however, the majority of cells had only undergone two or three divisions (Fig. 5, *d* and *e*). IL-4 and IL-13 reduced the proportion of CD40L-stimulated CB B cells that remained undivided, resulting in a greater proportion of cells in divisions two and three (Fig. 5*e*). In contrast, IL-10 not only decreased the proportion of undivided B cells, but also increased the proportion of cells in later divisions. Consequently, following stimulation with CD40L and IL-10, the greatest proportions of cells were found in divisions three and four. IL-21 had a similar effect as IL-10, however, virtually no cells were detected in divisions zero to two, and the majority had undergone six or more rounds of cell division (Fig. 5, *d* and *e*). When IL-21 was combined with IL-4 or IL-10, the distribution of CD40L-stimulated B cells across division number was similar to that observed for IL-21 alone (Fig. 5*f*), consistent with the results when proliferation was quantitated by assessing [^{3}H]thymidine uptake (Fig. 5*c*). Therefore, in addition to IL-21 enhancing proliferation of mature splenic B cells, this cytokine is capable of activating neonatal B cells and it does so to a much greater extent than any other cytokine examined.

IL-21 causes extensive expansion of the GC B cell population

Having established the dynamics of responses of naive splenic and CB B cells as well as memory B cells to IL-21, we next examined the effect of IL-21 on GC B cells. These cells are highly susceptible to apoptosis (3, 55); consequently, if they are to differentiate into PC or memory B cells they require survival and proliferative signals which include CD40L, Ag, and/or T-derived cytokines (3, 22, 55). Th_F cells localize to GC and are the predominant source of IL-21 (26, 27, 29). Because T_{FH} cells provide instructive signals to GC B cells (27), we examined the effect of IL-21 on the expansion of GC B cells. GC B cells expressed IL-21R (Fig. 5*g*). GC B cells represent B cells that have been activated in vivo (55). Thus, expression of IL-21R by these cells is consistent with increased expression of IL-21R on in vitro-activated human B cells (Fig. 2). When cultured for 4 days in vitro, <5% of unstimulated GC B cells survived the culture period (Fig. 5*h*), a finding consistent with previous studies (55). However, their viability could be improved 50-fold by addition of CD40L (Fig. 5*h*), and by a further ~1.5-fold in the presence of IL-10 (Fig. 5*h*). In contrast, IL-21 exhibited a much greater effect on proliferation of GC B cells by expanding the numbers of these cells 4- to 5-fold compared with CD40L alone (Fig. 5*h*). The data from experiments that examined the recovery of viable GC B cells were consistent with those obtained when proliferation was assessed by incorporation of [^{3}H]thymidine. Under these conditions, IL-2 and IL-15 had negligible effect on proliferation of CD40L-stimulated GC B cells (data not shown), while IL-4- and IL-10-enhanced proliferation by up to 2-fold (Fig. 5*i*). However, the effect of IL-21 was >5-fold. When CD40L-stimulated GC B cells were cultured with both IL-10 and IL-21, no further increment in proliferation was observed (Fig. 5*i*). Therefore, akin to neonatal CB B cells as well as splenic naive and memory B cells, CD40L-treated GC B cells also undergo extensive proliferation in the presence of IL-21, a response that greatly exceeds that induced by other cytokines with B cell stimulatory characteristics.

Cytokine secretion by B cell subsets in response to IL-21

Little is known regarding the mechanism by which IL-21 induces such potent responses in human B cells. Production of cytokines by B cells stimulated through CD40 or the BCR can contribute to the ability of these stimuli to induce B cell responses (56). To investigate whether IL-21 promoted secretion of cytokines by B cells, splenic naive, memory, and tonsillar GC B cells were stimulated with CD40L alone or with IL-21 or anti-Ig, following which the production of IL-6 and IL-10 was measured. Naive and memory B cells produced IL-6 when stimulated with CD40L, and the amounts produced were increased by the inclusion of anti-Ig, but not IL-21, in the culture (Fig. 6*a*). In contrast to IL-6, production of IL-10 by CD40L-stimulated naive and memory B cells was below the level of detection (Fig. 5*b*). However, IL-10 was secreted by all B cell subsets when they were cultured with CD40L and IL-21, with memory B cells producing 2- to 3-fold more IL-10 than naive B cells. Nonetheless, the amount of IL-10 produced in the presence of IL-21 remained less than that induced by CD40L and anti-Ig (Fig. 6*b*). GC B cells failed to produce IL-6 when stimulated with CD40L alone or in combination with IL-21 (data not shown), a finding consistent with previous studies (51). GC B cells could produce IL-10 in response to stimulation with CD40L alone (19 ± 4.3 pg/ml; $n = 2$ experiments; duplicate samples per experiment) and this response was increased 2-fold by IL-21 (40.5 ± 7.5 pg/ml). Thus, in addition to enhancing their proliferation, IL-21 can improve production of IL-10 by human B cells.

IL-21 induces phosphorylation of STAT3 in human B cells

Signaling through the IL-21R/γ_c complex results in activation of the JAK/STAT pathway and specifically involves STAT3 (24, 25). STAT3 has been found to be phosphorylated in myeloma cell lines and B-CLL cells exposed to IL-21 in vitro (54, 57), however, no studies have examined the effects of IL-21 on STAT3 activation in primary human B cells. For these experiments, purified splenic B cells were initially cultured in the absence or presence of IL-21 for different periods of time. Human B cells do not express detectable

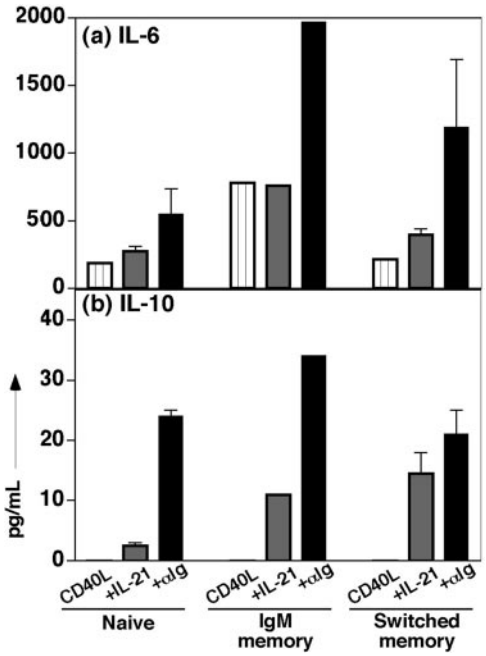

FIGURE 6. Cytokine secretion by B cell subsets. Sort-purified naive, IgM memory, and switched memory B cells were cultured with CD40L alone (▥) or in combination with IL-21 (50 ng/ml; ▨) or anti-Ig (10 μg/ml; ■). After 4 days, secretion of (*a*) IL-6 and (*b*) IL-10 was determined by cytokine-specific immunoassays. The data are the mean ± SEM and represent at least three experiments performed using cells from different donors.

levels of phospho-STAT3 when examined immediately following purification (Fig. 7*a*). When expression of phospho-STAT3 was assessed in IL-21-stimulated B cells, no change was detected after 5 min compared with cells cultured but unstimulated for the same time (Fig. 7*b*). However, there was a weak but consistent induction in expression of phospho-STAT3 after 15 min stimulation as revealed by a ~2-fold increase in the MFI of phospho-STAT3 in cells treated with IL-21 compared with unstimulated controls (Fig. 7*c*). As a positive control, the myeloid cell line U937 was stimulated with IL-6, which is known to induce STAT3 phosphorylation. IL-6 increased expression of phospho-STAT3 3- to 5-fold (Fig. 7*d*), indicating that although the effect of IL-21 on expression of phospho-STAT3 in human B cells was weak, it was in the range of that observed for an established cell line. Thus, IL-21 most likely enhances responses of CD40L-stimulated human B cells by activating a STAT3-dependent signaling pathway.

Discussion

Naive and memory B cells underlie primary and secondary humoral immune responses, respectively, and react differently to certain types of stimuli. Memory B cells respond more robustly than naive B cells in vitro to extrinsic stimuli such as CD40L, TLR ligands, and cytokines, as demonstrated by a shorter ttfd, greater proliferation, the generation of a greater number of plasmablasts, and increased Ig secretion (8, 10–14). IL-21 has recently been recognized as a cytokine capable of inducing proliferation of human and murine B cells (23, 30, 32, 33). Although a recent study presented data on the responses to IL-21 of naive B cells in CB and memory B cells in peripheral blood (PB), these cells were from different donors and tissues, hence quantitative comparisons of the responses could not be made (30). Therefore, no studies have systematically examined the kinetics of proliferation of naive and memory B cells from the same tissue in response to IL-21. Similarly, the response of GC B cells, which, due to their location, are most likely to be exposed to the greatest concentrations of IL-21 produced by Th_F cells (26, 27, 29), to IL-21 remains unexplored. For these reasons, we sought to examine expression of IL-21R within the B cell lineage and to provide an in-depth comparison of B cell subset behavior in response to IL-21 vs other well-characterized B cell growth factors.

IL-21R is expressed on a wide range of lymphoid cells (23, 33). By examining different subsets of human B cells by microarray analysis, sq-PCR and flow cytometry, IL-21R was shown to be present on naive B cells in spleen, CB, and PB, tonsillar GC B cells, but neither memory B cells nor PC (Figs. 1 and 5). Human myeloma cell lines express IL-21R, and IL-21 acts as a survival factor for these cells in vitro (57). Thus, sustained expression of IL-21R on malignant, but not normal, PC may represent a mechanism whereby myeloma cells have a survival advantage in vivo. Although memory B cells lacked expression of IL-21R, it was rapidly induced following activation in vitro (Fig. 2). This presumably underlies the ability of memory B cells, as well as corresponding naive B cells, to exhibit robust proliferation in response to IL-21 (Figs. 3 and 4; Ref. 30).

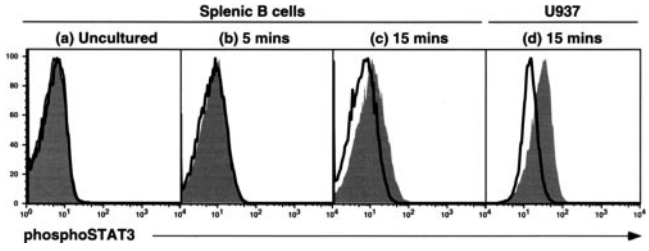

FIGURE 7. IL-21 induces phosphorylation of STAT3 in human B cells. Splenic B cells were assessed for expression of phospho-STAT3 immediately following purification (*a*) or after 5 (*b*) or 15 (*c*) min of stimulation with IL-21 (100 ng/ml). As a positive control, U937 cells were stimulated with IL-6 (50 ng/ml) for 15 min (*d*). In all plots, the solid gray histogram represents expression of phospho-STAT3 in cells treated with the indicated stimulus. In *a*, the overlay outline histogram represents fluorescence of cells incubated with a control mAb. In *b–d*, the overlay histogram represents expression of phospho-STAT3 in cells cultured for the same period of time in the absence any stimulus.

When we examined the effect of IL-21 on B cell proliferation, our findings extended previous reports (23, 30) by demonstrating that IL-21 enhanced CD40L-induced proliferation of human B cells present in spleen (naive, memory; Figs. 3 and 4), tonsil (GC; Fig. 5), and CB (Fig. 5). Furthermore, the magnitude of proliferation of the B cell subsets induced by the combination of CD40L and IL-21 was substantially greater, and the ttfd of naive and memory B cells was significantly less than that induced by all other cytokines (IL-4, IL-10, IL-13, IL-15) examined. We found that the proliferation of B cells, irrespective of their stage of differentiation, induced by CD40L and IL-21 was not further increased by including IL-4, IL-10, or IL-13 in the culture (Fig. 5; data not shown). At face value, these findings may appear to contradict recent findings that IL-4, IL-13 (58), and IL-10 (59) could enhance proliferation of human PB B cells stimulated with anti-CD40 mAb and IL-21. There are at least three explanations for these discrepancies. First, the studies by Caven et al. (59) and Wood et al. (58) used PB B cells, while our study used B cell purified from lymphoid tissues (spleens, tonsils). However, this is unlikely to be the sole reason because we also failed to observe any combined effect of IL-21 and other B cell stimulatory cytokines on proliferation of CD40L-stimulated CB B cells. A second, and more plausible, explanation is that these other studies used either total PBMC (59), or enriched populations of B cells, however, the purity was <90% (58). In contrast, we isolated B cell subsets by sorting such that the purity was usually >97%. Thus, because IL-21 has pleiotropic effects on other cell types, such as T cells and NK cells (23, 25, 60, 61) that would still be present in these cultures, the apparent increase in anti-CD40 mAb-induced B cell proliferation observed in the presence IL-4, IL-10, or IL-13 may result from an indirect effect of IL-21 on T cells and NK cells. Similarly, IL-4, IL-10, and IL-13 can all influence the behavior of monocytes, with respect to phenotype and cytokine secretion (62). Thus, residual monocytes present in cultures of human PBMC stimulated with these cytokines may modulate the B cell response. Indeed, monocytes stimulated with IL-10 have been found to be capable of regulating Ig secretion by cocultured B cells (63). The third possibility is the nature of the stimulating agent, as we used recombinant multimeric CD40L, while Caven et al. (59) and Wood et al. (58) both used anti-CD40 mAb. Overall, our results highlight the importance of using highly purified populations of lymphocytes to reveal the direct effects of cytokines on lymphocyte behavior.

The net stimulatory effect of IL-21 on CD40L-stimulated naive B cells exceeded that for memory B cells, as indicated by a greater stimulation index relative to CD40L alone, and sustained and maximal proliferation of naive cells over an extended period of time (48–72 h) compared with memory B cells (24 h). The response of naive B cells to CD40L and IL-21 was comparable to that induced by CD40L and anti-Ig, a stimulus we have found to induce the greatest level of B cell proliferation in vitro. In contrast, the response of memory B cells to CD40L and IL-21 was ~50% of that achieved with CD40L and anti-Ig. This difference in the response of naive and memory B cells to IL-21 parallels the expression of at least twice as much IL-21R on the surface of naive B cells than

on memory B cells following 48 h of activation (Fig. 2). This may contribute to the ability of IL-21 to stimulate naive B cells to their full potential, allowing them to proliferate to the same extent as memory B cells in in vitro culture, an outcome not previously seen with other B cell stimuli. It is important to appreciate that during the early stages of in vitro activation (~12 h), IL-21R expression increased only on the memory B cell subset, providing an explanation as to why naive B cells were not more sensitive to IL-21 (Fig. 4), despite basal expression of IL-21R on resting naive B cells (Fig. 1). Furthermore, although IL-21 induced a greater response of naive B cells relative to memory B cells when compared with the effects of CD40L alone (i.e., fold increase, frequency of cells recruited into division; see Figs. 3 and 4), memory B cells still entered division earlier, and more of these cells were recruited into division than naive B cells, irrespective of the extrinsic stimuli used. This appears to be due to an intrinsic threshold set for individual populations that regulates the maximal response attained by in vitro stimulation (K. L. Good and S. G. Tangye, manuscript in preparation). In addition, the pronounced effect of IL-21, coupled with an intrinsic earlier ttfd of memory B cells, may result in an accelerated exhaustion of memory B cell cultures and hence the more notable change in the proliferative characteristics of naive B cells.

IL-21 also induced CD40L-stimulated naive, GC, and memory B cells to secrete increased amounts of IL-10 compared with that induced by CD40L alone (Fig. 6). This is consistent with the recently reported ability of IL-21 to increase secretion of IL-10 by human PBMC stimulated with anti-CD40 mAb (58) and cytokine-stimulated human and mouse NK cells (60, 61), respectively. However, the same caveats apply to the effects of IL-21 on cytokine secretion by CD40-stimulated human PBMC as those detailed above for proliferation, because it is possible that monocytes, which constitutively express CD40 and respond to activation with CD40L by secreting cytokines (64), were responsible for the IL-10 secretion noted in these cultures (58).

For B cells to differentiate into PC, they must first undergo several rounds of cell division (12, 65, 66). Thus, the potency of IL-21 on proliferation of both naive and memory B cells is consistent with the reported production of large quantities of Ig by human PB B cells following stimulation with anti-CD40 mAb and IL-21 (30). However, this study only compared IgG secretion by separated PB naive and memory B cells that were stimulated with anti-CD40 and anti-Ig Ab together with IL-2 and IL-21. Thus, it is currently unclear whether IL-21 alone has a distinct effect on the differentiation of naive and memory B cells into PC. This issue is currently being addressed (V. L. Bryant and S. G. Tangye, manuscript in preparation).

The mechanism of action of IL-21 on lymphocytes involves the JAK1 and JAK3 kinases which, following recruitment to IL-21R and γ_c, respectively, phosphorylate members of the STAT family of transcription factors, including STAT3 (25). We found that STAT3 became phosphorylated in splenic B cells following stimulation with IL-21 (Fig. 7). This is the first demonstration of IL-21-induced activation of STAT3 in primary human B cells, and extends previous studies that presented similar findings for malignant or transformed human B cells (54, 57). These results suggest that signaling pathways triggered by the dual activation CD40 and IL-21R complex would synergize to induce a potent response in human B cells.

IL-21 has distinct effects on B cell proliferation, dependent on the concurrently applied stimuli. For instance, proliferation of human or murine B cells induced by CD40L or anti-Ig, respectively, was augmented by IL-21 (Refs. 23, 30, 33; Fig. 2), while IL-21 suppressed proliferation induced by LPS (murine; Ref. 33) or anti-Ig plus IL-4 (human; Ref. 23) by inducing apoptosis (32, 33). More recently, IL-21 was reported to deliver proapoptotic signals to B-CLL cells activated with CD40L (54). Accordingly, it was proposed that IL-21, in combination with CD40L, may be a useful therapeutic to reduce the burden of leukemic cells in B-CLL patients (54). Although the vast majority of B cells in these patients belong to the malignant clone, our data indicate that administration of CD40L and IL-21 would result in considerable activation and expansion of the normal residual B cells, a potentially unwanted outcome. Despite this, the potency of IL-21 on proliferation of B cells indicates that it may have clinical application in settings of immunodeficiency. For example, there is a paucity of memory B cells in immunocompromised individuals, such as those recovering from stem cell transplantation (43, 67), or patients with immunodeficient states (15, 34–36, 41). Thus, careful administration of IL-21 has the potential to restore the B cell compartment of these patients.

In contrast, excessive production of IL-21 by Th_F cells, and its subsequent effects on B cells, may perturb lymphocyte homeostasis in vivo. IL-21 is overproduced in murine models of SLE, namely the *san roqin* (29) and BXSB-Yaa (28) strains of mice. Moreover, T_{FH} cells are also increased in *san roqin* mice (29). Another feature of T_{FH} cells is elevated expression of ICOS (27, 29). An increase in $CD4^+ICOS^+$ T cells has been detected in the PB of some SLE patients (42). Thus, Th_F cells, and serum levels of IL-21, may also be elevated in lupus patients. A characteristic of SLE patients is a reduction in the numbers of naive and memory B cells in their PB, coupled with the presence of large numbers of GC-founder cells (39, 40), cells that are highly prone to apoptosis and are usually restricted to lymphoid tissues (68). Because IL-21 can promote activated GC B cells to undergo expansion and secrete IL-10, a known antiapoptotic factor for such cells (69), it is possible that IL-21 may support the survival and expansion of GC-founder cells in the PB of SLE patients. Interestingly, IL-10 is also elevated in the serum of SLE patients due to dysregulated production by B cells and monocytes, rather than by T cells (37). Therefore, another potential contribution of IL-21 to autoimmune conditions like SLE would be to increase production of regulatory cytokines, such as IL-10, by pathogenic B cells (38). This proposition is supported by the findings here that IL-21 did not enhance production of IL-6 by human B cells, and that IL-6 does not contribute to B cell dysfunction in human SLE (37). For these reasons, it would be of interest to measure the levels of IL-21 in serum of patients with Ab-mediated autoimmune conditions.

In summary, our findings have shed new light on the role of IL-21 as a growth factor for human B cell subsets. IL-21 greatly increased proliferation of CD40L-stimulated B cells irrespective of their stage of differentiation and to a greater extent than all other cytokines examined. Our data support the proposal by Lipsky and colleagues (30) that IL-21 may have therapeutic use to enhance recovery of the B cell compartment in immunodeficiency states, whereas its inhibition may improve disease outcome in autoimmune conditions like SLE, where there is marked perturbations to B cell homeostasis.

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Disclosures

The authors have no financial conflict of interest.

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