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IL-21-Induced Isotype Switching to IgG and IgA by Human Naive B Cells Is Differentially Regulated by IL-4

Danielle T. Avery, Vanessa L. Bryant, Cindy S. Ma, Rene de Waal Malefyt, and Stuart G. Tangye

Naive B cells can alter the effector function of their Ig molecule by isotype switching, thereby allowing them to secrete not only IgM, but also the switched isotypes IgG, IgA, and IgE. Different isotypes are elicited in response to specific pathogens. Similarly, dysregulated production of switched isotypes underlies the development of various diseases, such as autoimmunity and immunodeficiency. Thus, it is important to characterize mediators controlling isotype switching, as well as their contribution to the overall B cell response. Isotype switching in human naive B cells can be induced by CD40L together with IL-4, IL-10, IL-13, and/or TGF-β. Recently, IL-21 was identified as a switch factor for IgG1 and IgG3. However, the effect of IL-21 on switching to IgA, as well as the interplay between IL-21 and other switch factors, remains unknown. We found that IL-4 and IL-21 individually induced CD40L-stimulated human naive B cells to undergo switching to IgG, with IL-4 predominantly inducing IgG1+ cells and IL-21 inducing IgG3. Culture of naive B cells with CD40L and IL-21, but not IL-4, also yielded IgA+ cells. Combining IL-4 and IL-21 had divergent effects on isotype switching. Specifically, while IL-4 and IL-21 synergistically increased the generation of IgG1+ cells from CD40L-stimulated B cells, IL-4 concomitantly abolished IL-21-induced switching to IgA. Our findings demonstrate the dynamic interplay between IL-4 and IL-21 in regulating the production of IgG subclasses and IgA, and suggest temporal roles for these cytokines in humoral immune responses to specific pathogens. The Journal of Immunology, 2008, 181: 1767–1779.

The primary function of B cells is to produce Ag-specific Ig. Naive B cells have the remarkable ability to alter the effector function of their Ig molecule by the process of isotype switching. Thus, naive B cells initially expressing IgM and IgD can secrete not only IgM, but also the switched isotypes IgG, IgA, and IgE (1–3). Isotype switching is usually induced in response to T-dependent Ag following interactions between Ag-primed naive B cells, Ag-specific CD4+ T cells, follicular dendritic cells, and dendritic cells (DC) (4–7). Isotype switching typically occurs following the receipt of two signals: CD40L and a specific cytokine (8, 9). The cytokines IL-4, IL-10, IL-13, IL-21, and TGF-β have been found to be switch factors for the in vitro production of IgG1, IgG2, IgG3, IgG4, IgA, and IgE by activated human naive B cells (10–21). More recently, the TNF family members BAFF (B cell-activating factor belonging to the TNF family) and APRIL (a proliferation-inducing ligand) have also been reported to induce isotype switching, as evidenced by the expression of Ig germline transcripts (GLTs) in naive B cells treated with either BAFF or APRIL, the levels of which were augmented by IL-4 or IL-10, as well as by secretion of IgG3 and IgA in response to stimulation with BAFF, anti-Ig, and the cytokines IL-2 or IL-15 (22, 23). The resulting IgG, IgA, or IgE molecules differ from one another in their abilities to cross mucosal surfaces, fix complement, and activate other effector cells through differential binding to FcR (1–3). Switching to downstream isotypes, therefore, provides versatility in both Ig function and distribution, without altering antigenic specificity, since the Ig variable region remains unchanged during this process.

The production of distinct Ig isotypes in humans is associated with immune responses to different classes of pathogens. Viral infections often result in production of IgG1 and IgG3; encapsulated bacteria (e.g., Streptococcus, Haemophilus) expressing polysaccharide-rich Ag elicit IgG1 and IgG2 responses; parasitic infections generate IgG4 and IgE; and pathogens that breach mucosal barriers induce IgA (1–3). The IgG subclass elicited can also change throughout an immune response. For example, during the acute phase of some infections, virus-specific IgG1 is initially detected, followed ~10 days later by IgG3. On the other hand, the anti-viral IgG response detected after acute infection is comprised of IgG1, but not IgG2 or IgG3 (24–27). Thus, it is important that the appropriate Ig isotype is induced following Ag exposure, such that the relevant mechanisms ensuring efficient Ag clearance are activated, thereby limiting and controlling infection.

Several human diseases are associated with the dysregulated production of isotype-switched Ab. Pathogenic Abs in patients...
with autoimmune conditions (e.g., systemic lupus erythematosus) are usually of the IgG isotype (28), while IgA may play a role in diseases such as celiac disease (29), IgA-mediated vasculitis (30), and IgA nephropathy (31). On the other hand, patients with deficiencies in specific Ig isotypes are susceptible to various infections. For example, patients with common variable immunodeficiency have reduced serum levels of IgG and IgA, and they suffer from recurrent pulmonary infections (32–34). Similarly, individuals deficient in IgG1 or IgG3 have frequent respiratory tract infections and increased viral infections, while IgG2 deficiency renders individuals poorly responsive to polysaccharide Ag and they are therefore prone to infection with encapsulated bacteria (1, 3). Lastly, selective IgA deficiency is the most common form of immunodeficiency (2, 32). Although most IgA-deficient patients are asymptomatic, some suffer from recurrent infections (2, 32). For these reasons, it is important to define the contributions of specific mediators of Ig isotype switching to the overall B cell response.

Recently, IL-21 was shown to induce human B cells to undergo isotype switching to IgG1 and IgG3 (19, 35). Despite these important findings, the effect of IL-21 on switching of human B cells has not been investigated. Herein, we have addressed the role of IL-21, together with IL-4 and IL-10, in regulating isotype switching to subclasses of IgG and IgA by human naive and IgM*CD27⁺ (i.e., IgM memory) B cells.

Materials and Methods

Monoclonal Abs

FITC-anti-CD20, FITC-anti-CD57, and PE-Texas Red anti-CD4 mAbs were purchased from BD Biosciences; biotinylated anti-human IgM, IgG1, IgG2, IgG3, IgG4, and IgA mAb, PE-anti-CD27 mAb, allophycocyanin-conjugated anti-IgG mAb, Alexa 647-anti-CXCR5, and streptavidin (SA) conjugated to PerCP (SA-PerCP) were purchased from BD Pharmingen. Biotinylated and allophycocyanin-conjugated anti-CD27 were purchased from eBioscience. Biotinylated anti-human IgD, IgA1, and IgA2 mAbs were from Southern Biotechnology Associates. Neutralizing anti-IL-4 (clone MP4-25D2) and anti-IL-10 (clone JES5-9D7) mAbs were provided by DNAX Research Institute (Palo Alto, CA). Neutralizing IL-21R-Fc was purchased from R&D Systems.

B cell isolation

Spleens from cadaveric organ donors and peripheral blood (PB) buffy coats were obtained from the Australian Red Cross Blood Service. Tonsils and umbilical cord blood (CB) samples were provided by the Royal Prince Alfred Hospital (Sydney, Australia). Mononuclear cells were isolated by centrifugation over Ficoll-Paque. B cells were isolated from mononuclear cells using a B cell-negative isolation kit (Dynal Biotech). Purified splenic B cells were fractionated into subsets of naive and IgM memory B cells according to their differential expression of CD27 and IgG isotypes (36–38). Thus, total B cells were labeled with anti-CD20 and anti-CD27 mAbs together with mAbs to IgG, IgA, and IgE. Naive and IgM memory B cells were defined as CD20⁺CD27⁺IgG IG A⁻ and CD20⁺CD27⁺IgG IG A⁺ cells, respectively (36, 38, 39). Although it is possible that engaging CD27 with a specific mAb may result in preferential activation of memory B cells, this is unlikely, as culture of human B cells with CD70-expressing cells alone failed to induce B cell proliferation or differentiation (40). Cells were sorted on a FACSAria flow cytometer (BD Immunohistochemistry Systems). Gating strategies excluded doublets. The purity for each population was typically >98%. All studies were approved by institutional human ethics review committees.

B cell cultures

Purified B cells were labeled with CFSE (Molecular Probes) (41, 42) and then cultured in the presence of recombinant human CD40L alone (prepared as membranes of insect cells infected with baculovirus expressing CD40L (39, 43) or with IL-4 (100 U/ml), IL-10 (100 U/ml; both provided by DNAX Research Institute), IL-21 (50 ng/ml; PeproTech), or IL-4 and IL-21. For phenotypic analysis, the cells were cultured in 48-well plates (~1–1.5 × 10⁵/400 μl/well; BD Labware) for 5–6 days. In some experiments, B cells were cultured in round-bottom 96-well plates (~20 × 10⁵/200 μl/well; BD Labware) for 10–12 days.

Immunofluorescent staining

In vitro-activated naive and IgM memory B cells were harvested from culture wells and incubated with isotype control, anti-IgG, or anti-IgA mAbs on ice. To determine expression of IgG and IgA subclasses, cells were incubated with biotinylated anti-IgG1, IgG2, IgG3, IgG4, IgA1, or IgA2 mAb; bound Ab was revealed by the addition of SA-PerCP. Data were acquired on a FACS Calibur using CellQuest software (BD Biosciences). Cells present in different divisions were characterized by “division slicing” (42, 44–48). The frequency of cells expressing different surface Ig isotypes was then determined by backgating and analyzing the differently divided populations as defined by CFSE dilution (42, 44–48) using FlowJo (Tree Star).

CD4⁺ T cell–B cell co-cultures

CD4⁺ T cells were isolated from human tonsils using CD4 DynaBeads (Dyna Biotech) (48), CD4⁺CXCR5⁺CD57⁺, CD4⁺CXCR5⁺CXCR5⁺, and CD4⁺CXCR5⁺CD57⁺ T cell subsets were isolated by sorting (49), treated with mitomycin C (40 μg/ml; Sigma-Aldrich), and then cultured in 96-well U-bottom tissue culture plates with autologous naive B cells (25 × 10⁵ each cell/200 μl/well) in the absence or presence of PHA (5 μg/ml; Sigma-Aldrich) and IL-2 (20 U/ml; Chemicon International) (48, 49). Endogenous IL-4, IL-10, or IL-21 was neutralized by anti-IL-4 or anti-IL-10 mAbs (20 μg/ml) or IL-21R-Fc (10 μg/ml; R&D Systems). Ig secretion was determined after 10–12 days by ELISA.

Ig and cytokine ELISAs

Secretion of IgM, IgG, and IgA was determined by Ig H chain-specific immunoassays (50). To measure IgG subclasses, 96-well microtiter plates (Thermo Electron) were coated with sheep anti-human IgG1, IgG2, and IgG3 polyclonal antisera (The Binding Site) or mouse anti-human IgG4 mAb (clone HP6025; Calbiochem). Nonspecific binding sites were blocked with 2% PCS/PBS. Culture supernatants and Ig subclass myeloma standards (Calbiochem) were added to the wells and incubated for 2 h at 37°C before adding HRP-conjugated sheep anti-human IgG1, IgG2, IgG3, or IgG4 polyclonal antisera (The Binding Site). Bound Ab was visualized with TMB substrate (Sigma-Aldrich: 0.1 mg/ml) either prepared in 0.1 M sodium acetate buffer (pH 6.0) containing 0.01% H₂O₂ or purchased as the OptiEIA TMB substrate reagent set (BD Biosciences). To examine cytokine secretion, sort-purified B cells (5–10 × 10⁶/ml) were cultured in 96-well plates with CD40L alone or in combination with IL-4, IL-21, or IL-4 plus IL-21. Supernatants were collected after 2 and 5 days, and secretion of IL-6 and IL-10 was measured by specific ELISAs (39, 51) using the TMB substrate outlined above for Ig ELISAs.

Semiquantitative PCR analysis: Ig GLT1s and cytokines

RNA was isolated from cultured CB B cells or sort-purified CD4⁺ T cells (Qiagen RNeasy kit), transcribed into cDNA, normalized for expression of the constitutively expressed gene GAPDH (5′-TGG TCG TAT TAT GCG GGC C, 3′-GOT CAT GAG TCC TTC CAC GAT ACC) and then used as a template for semiquantitative PCR, as previously described (39, 52). The following primers were used (Sigma-Genosys): Ig GLTs: IgG, 5′-GGG CTT CCA AGC CAA CAG GGC AGG ACA; Cγ₁, 5′-GTT TTG TTA CAG GAT GGC TTC C, 3′-GTT GGC ACT CGA CAA ACC TCT TGC G; Cγ₅, 5′-AGG TGG GCA GCA GGC TTC AGG CCC CGAT C; Cγ₂, 5′-TTG TGG TCA GAC GCA GCC TGC A; Cγ₃, 5′-AGG TGG GCA GCA GGC TTC AGG CCC CGAT C; Cα₁, 5′-GGG TGG GCA GGG TTA GGG TCT TGG; Cα₂, 5′-TGG TGG GCA GGG TTA GGG TCT TGG; Cα₅, 5′-TGG TGG GCA GGG TTA GGG TCT TGG; Cα₆, 5′-TGG TGG GCA GGG TTA GGG TCT TGG.

Cytokines: IL-4, 5′-CAG CAG CCC TCT TGG CAG GCA GCC AG; Cα₁, 5′-GGG TGG GCA GGG TTA GGG TCT TGG; Cα₂, 5′-TGG TGG GCA GGG TTA GGG TCT TGG; Cα₅, 5′-TGG TGG GCA GGG TTA GGG TCT TGG; Cα₆, 5′-TGG TGG GCA GGG TTA GGG TCT TGG.

Results

IL-21 induces human CD40L-activated naive B cells to undergo division-linked Ig isotype switching to IgG: synergistic effect of IL-4

It has been previously reported that mouse and human naive B cells undergo division-linked Ig isotype switching, as determined by detection of expression of surface IgG and IgA, when cultured

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with CD40L and different cytokines (42, 44–47). The culture system used previously to examine these events in IL-4-treated human naive B cells (42, 48) was used to compare the effects of IL-4 and IL-21 on isotype switching. CFSE-labeled naive B cells from human spleen, PB, or CB were cultured with CD40L in the absence or presence of IL-4 or IL-21. After 5 days, expression of surface IgG was determined (Fig. 1). CD40L-stimulated naive B cells did not undergo isotype switching, as <0.5% of cells in these cultures were IgG⁺ (Fig. 1a). However, IgG⁺ cells were detected in cultures of B cells stimulated with CD40L and IL-4 (Fig. 1b) or IL-21 (Fig. 1c) irrespective of the source of the cells. In general, greater frequencies of IgG⁺ cells were detected following culture with CD40L/IL-21 than with CD40L/IL-4 (Fig. 1, b and c). Since IL-4 and IL-21 both induced switching to IgG and signal through γc (56), the effect of combining these cytokines was also examined. IL-4 together with IL-21 had a synergistic effect on switching to IgG, with the frequencies of IgG⁺ cells generated exceeding that induced by the cumulative effect of each cytokine individually by ~2-fold (Fig. 1d).

Division slicing of CFSE peaks was used to assess the rate of switching to IgG on a division basis. Very few, if any, IgG⁺ cells were found in any division of naive B cells stimulated with CD40L alone (Fig. 1e, □). However, when cultures were supplemented with IL-4 or IL-21, naive B cells underwent isotype switching to IgG in a division-linked manner (Fig. 1e, △, ○). The division-linked rate of switching to IgG for splenic, PB, and CB naive B cells in response to CD40L and either IL-4 or IL-21 was similar, which is consistent with the similar proportions of IgG⁺ cells observed in each culture condition at the bulk population level (Fig. 1, b, c, and e). A greater proportion of IgG⁺ B cells was detected in each division of cells stimulated with CD40L/IL-4/IL-21 compared with those stimulated with CD40L/IL-4 or CD40L/IL-21 (Fig. 1e, △). This analysis demonstrated that the synergistic effect of IL-4 plus IL-21 on switching by CD40L-stimulated B cells at the bulk population (Fig. 1d) did not result from greater proliferation since dilution of CFSE by CD40L/IL-21- and CD40L/IL-4/IL-21-stimulated B cells was similar (Figs. 1, c and d). We also investigated proliferation by assessing incorporation of [³H]thymidine by CB B cells stimulated with CD40L alone or in the presence of IL-4, IL-21, or IL-4 and IL-21. The findings from this analysis supported the CFSE data, such that addition of IL-4 to cultures of B cells stimulated with CD40L/IL-21 did not cause a further augmentation in proliferation over that induced by CD40L/IL-21 (data not shown; Ref. 39). Taken together, these findings demonstrate that more IgG⁺ cells were generated per division, indicating that the combination of these cytokines enhances the rate of isotype switching, not proliferation. Overall, these results revealed that IL-4 and IL-21 induce isotype switching in CD40L-stimulated human naive B cells to IgG at a comparable rate and that the combination of these cytokines has a synergistic outcome on this event.

**IL-4 and IL-21 have distinct effects on the ability of CD40L-stimulated naive B cells to switch to IgG subclasses**

The effects of IL-4 on switching to IgG subclasses are unclear, because numerous studies have found that IL-4 induces either...
expression of only $\gamma_4$ GLT and secretion of IgG4 by CD40-stimulated human B cells (10, 57, 58), induces expression and production of multiple IgG subclasses (53–55, 59, 60), or has little effect on secretion of IgG subclasses over that induced by CD40 engagement (19, 21). In contrast, IL-21 exclusively promotes expression of $\gamma_1$ and $\gamma_3$ GLTs, as well as secretion of IgG1 and IgG3 (19).

Because of the conflicting results regarding IL-4, and our observation that IL-4 and IL-21 synergized to increase the rate of switching to IgG by CD40L-stimulated B cells, we determined the individual and combined effects of IL-4 and IL-21 on the acquisition of expression of IgG subclasses. When splenic naive and CB B cells were stimulated with CD40L/IL-4, $\sim$55% of the cells that acquired surface IgG expressed IgG1, while the remainder were IgG2$^+$ ($\sim$22%) or IgG3$^+$ ($\sim$18%), and $<5\%$ were IgG4$^+$ (Figs. 2, a and b, and 3a). In contrast, $\sim$70% and 80%, respectively, of IgG$^+$ cells from cultures of splenic naive and CB B cells stimulated with CD40L/IL-21 were IgG3$^+$, $\sim$10–20% were IgG1$^+$, while very few IgG2$^+$ (6–8%) and no IgG4$^+$ cells were generated (Figs. 2, a and b, and 3a). When division-linked switchings of CD40L-stimulated CB B cells to total IgG and subclasses were compared, it was clear that the predominant subclasses induced by IL-4 and IL-21 were IgG1 and IgG3, respectively (Fig. 2, c and d).

The combination of IL-4 and IL-21 yielded predominately IgG1$^+$ cells from both splenic naive and CB B cells ($\sim$60% of IgG switched cells), as well as comparable frequencies of IgG2$^+$ and IgG3$^+$ cells (Figs. 2, a and b, and 3a). Thus, when used in combination, IL-4 had a dominant effect over IL-21, as evidenced by the increase in IgG1$^+$ and decrease in IgG3$^+$ B cells compared with those induced by IL-21 alone (Figs. 2, a and b, and 3a). This dominant effect of IL-4 was also apparent from analysis of the
Naive and IgM memory B cells exhibit different distributions of IgG subclasses following stimulation with CD40L and IL-21.

Human memory B cells comprises IgG^+ and IgA^+ cells, as well as those that continue to express IgM (reviewed in Ref. 61). The ability of IgM memory cells to undergo isotype switching has not been assessed. Thus, we compared isotype switching and secretion by naive and IgM memory B cells isolated from the same donor spleens following stimulation with CD40L and IL-4 and/or IL-21. The frequency of CD40L-stimulated IgM memory B cells that became IgG^+ following culture with IL-4, IL-21, or IL-4/IL-21 was ~50–75% less than naive B cells (data not shown). Despite this reduction it was still possible to analyze expression and secretion of IgG subclasses by stimulated IgM memory B cells and compare the response to naive cells. Similar to naive B cells, the IgG^+ cells appearing in cultures of CD40L- and CD40L/IL-4-stimulated IgM memory B cells comprised cells expressing predominantly IgG1, followed by IgG2 and IgG3 (compare Fig. 3, a and b, left panel), and low to negligible amounts of secreted IgG were detected (Fig. 3, a and b, right panel). In contrast to its effect on naive B cells, IL-21 did not preferentially induce expression or secretion of IgG3 by CD40L-stimulated IgM memory B cells (compare Fig. 3, left and right panels). Rather, the predominant subclass expressed was IgG1, followed by IgG2 and IgG3, thus resembling the effect of IL-4 on

We also measured secretion of IgG subclasses by CD40L-stimulated naive B cells isolated from spleen, PB, and CB. Culture of naive B cells with CD40L with or without IL-4 failed to induce substantial quantities of any IgG subclasses (Fig. 2f–h). However, secretion of IgG subclasses by CD40L-stimulated B cells was evident in the presence of IL-21: splenic and PB naive B cells secreted IgG3 and IgG1, and some IgG2 (Fig. 2, f and g), while CB B cells secreted predominantly IgG3 (Fig. 2h). IL-21 did not induce IgG4 secretion (Fig. 2f–h). The combination of IL-4 and IL-21 altered IgG secretion by CD40L-stimulated B cells, relative to the effect of these cytokines when used singly, such that IgG1 was the predominant isotype. IgG2 and IgG3 were produced in comparable quantities, and low amounts of IgG4 were detected (Fig. 2f–h). These data for IgG subclass secretion are generally consistent with those obtained for acquisition of expression of surface IgG1–4, inasmuch that the skewing to expression and production of IgG3 noted with IL-21 is attenuated by IL-4.
both naive and IgM memory B cells (Fig. 3, left panels). Consistent with this, IgG1, IgG2, and IgG3 were produced in similar amounts by IgM memory B cells in response to CD40L/IL-21 (Fig. 3b, right panel). The combination of IL-4 and IL-21 increased the proportion of CD40L-stimulated IgM memory B cells that expressed IgG2; this was at the expense of IgG1 (Fig. 3b, left panel). A similar effect was noted for IgG2 secretion by IgM memory B cells stimulated with CD40L/IL-4/IL-21 (Fig. 3b, right panel).

Thus, although IL-21 clearly induces preferential switching of naive B cells to IgG3 (Figs. 2 and 3a) (19), its effect on expression of IgG subclasses by IgM memory B cells mirrored that of IL-4. Furthermore, the combination of IL-4 and IL-21 resulted in the expression and production of substantial quantities of IgG2 by CD40L-activated IgM memory B cells.

IL-21 induces CD40L-activated naive B cells to undergo division-linked Ig isotype switching to IgA: opposing modulatory effects of IL-4 and IL-10

While the effect of IL-21 on isotype switching to IgG subclasses has been the subject of several reports (19, 35), and is extended by our data presented above, the consequences of culturing human naive B cells with IL-21 on switching to IgA have not been investigated. Thus, we examined IgA expression and secretion by B cells stimulated with CD40L and IL-21 and assessed the interplay between IL-21 and IL-4 or IL-10 on this process.

Culture of CB B cells with CD40L alone or with IL-4 did not induce switching to IgA (Fig. 4, a and b). However, adding IL-21 to CD40L-stimulated CB B cells resulted in the appearance of a
small population of cells that acquired expression of surface IgA (2–4% of cells; Fig. 4e). Similar findings were also obtained when naive PB B cells were examined (~2% IgA+ cells; data not shown). The vast majority of IgA+ cells (>75%) expressed the IgA1 subclass (Fig. 4e). Although IL-4 did not induce IgA expression by naive B cells (Fig. 4b), it abrogated the generation of IgA+(and IgA1+) B cells mediated by CD40L/IL-21 (Fig. 4, d and f). The effects of IL-21 and IL-4 on isotype switching to IgA were also evident from analysis of the rate of switching per division. CD40L/IL-21 induced a discrete population of naive B cells to express IgA (and IgA1) after they had undergone two divisions (Fig. 4, g and h). In contrast, the division-linked rate of switching to IgA characteristic of B cells stimulated with CD40L/IL-21 was dramatically attenuated in the presence of IL-4, such that it resembled cells stimulated with CD40L alone, that is, up to 10-fold fewer IgA+ cells generated per division with CD40L/IL-4/IL-21 compared with CD40L/IL-21 (Fig. 4, g and h). These findings were confirmed by assessing IgA secretion. Culture with CD40L or CD40L/IL-4 induced minimal secretion of IgA, while IL-21 resulted in the production of high levels of IgA (Fig. 4i). However, IL-4 reduced CD40L/IL-21-induced IgA secretion by ~5-fold (Fig. 4i).

IL-10 can induce or enhance IgA production by activated human B cells (15–17). It was therefore of interest to determine whether IL-10 could augment the effects of IL-21 on isotype switching to, and secretion of, IgA. IL-10 did not affect the frequency of IgA+ B cells generated from CB B cells stimulated with CD40L and IL-21 (Fig. 4j), nor the division-linked rate of switching (data not shown). However, IL-10 did increase the amount of IgA secreted by CD40L/IL-21-stimulated B cells (Fig. 4k). The effect of IL-10 onCD40L/IL-21-induced IgA secretion was only apparent after 8 days of culture (Fig. 4i). This may explain the lack of an effect of IL-10 on IL-21-induced switching to IgA when assessed by acquisition of IgA expression by B cells stimulated for only 5 days. Overall, these results reveal that IL-21 can induce isotype switching by CD40L-stimulated human B cells to IgA, and this process can be positively and negatively regulated by IL-10 and IL-4, respectively.

Expression of Ig H chain GLTs by stimulated B cells

Cytokines initiate isotype switching by inducing expression of Ig H chain GLTs (2, 5, 8). Thus, we were interested in determining the effect of IL-21 on expression of GLTs that encode IgG and IgA subclasses. CB B cells were cultured for 5 days in medium alone or with IL-4, IL-21, IL-4/IL-21, or CD40L alone or with these combinations of cytokines. IL-4 alone induced or increased expression of Igγ1, γ2, γ3, and γ4 GLTs (Fig. 5a–d, lane 2). CD40L had a similar effect to IL-4; however, it did not induce γ1 GLT (Fig. 5a–d, lane 5). CD40L was also capable of inducing Igα1 and Igα2 GLTs (Fig. 5, e and f, lane 5; Ref. 23). In contrast, IL-21 alone (Fig. 5a–d, lane 3) or in combination with IL-4 (lane 4), CD40L (lane 7), or CD40L/IL-4 (lane 8) had no effect on the basal or induced expression of any of the IgH chain GLT. In contrast to Igγ GLTs, IL-21 did appear to increase expression levels of Igα1 and Igα2 GLTs (Fig. 5, e and f, lane 7). Thus, although induction of GLTs is a prerequisite for Ig isotype switching (2, 5, 8), IL-21 was largely incapable of inducing expression of IgG GLTs in naive B cells. In this case, IL-21 is likely to facilitate isotype switching to IgG by enhancing proliferation of B cells that acquire expression of the appropriate GLT in response to stimulation with CD40L. On the other hand, IL-21 may mediate switching to IgA by increasing expression of Igα GLT induced by CD40L.

Cytokine production by human B cells stimulated with IL-4 and IL-21

Autocrine production of cytokines such as IL-6, IL-10, TNF-α, and TGF-β by in vitro-stimulated B cells has been found to contribute to their proliferation and differentiation induced by CD40 and/or BcR agonists (18, 63–65). We previously reported that splenic naive and memory B cells produced measurable amounts of IL-10 following stimulation with CD40L and IL-21, but not with CD40L alone (39). Since IL-4 and IL-21 had such diverse effects on isotype switching by human B cells, we were interested in determining the effect of these cytokines on the secretion of B cell stimulatory cytokines by CB B cells. CB B cells produced low-to-variable amounts of IL-6 and IL-10 in response to CD40L alone (Table I). However, consistent with previous studies (65), addition of IL-4 to cultures of CD40L-stimulated CB B cells resulted in marked production of IL-6, but not IL-10 (Table I). On the other hand, CD40L together with IL-21 induced secretion of detectable amounts of IL-10 and generally had little effect on IL-6 (Table I). In some experiments, the combination of CD40L, IL-4, and IL-21 increased production of IL-6 and IL-10 to levels that exceeded those induced by either cytokine alone (Table I). Thus, IL-4 and IL-21 are capable of inducing a specific pattern of cytokine production by CB B cells, with IL-4 predominantly inducing IL-6, and IL-21 inducing IL-10. CB B cells failed to produce detectable amounts of TNF-α in response to CD40L with or without these cytokines (data not shown).

CD4+ CXCR5+ T follicular helper (Tfh) cells induce isotype switching and Ig production by naive B cells: roles of endogenous IL-4, IL-10, and IL-21

At least three subsets of tonsillar CD4+ T cells can be identified by the differential expression of CXCR5 and CD57 (Fig. 6a) (66). These cells differ with respect to their ability to produce cytokines and provide B cell help (49, 66–68). The CD4+CXCR5+ subset, termed Tfh cells, is the predominant subset capable of inducing differentiation of B cells into Ig-secreting cells (49, 66–68). We have recently reported that this effect is largely mediated by production of IL-21 (49). Kuchen...
and colleagues also found that PB CD4⁺ T cells induce IgM and IgG secretion by B cells in an IL-21-dependent manner (69). Thus, we were interested in comparing the ability of subsets of tonsil CD4⁺ T cells to induce naive B cells to produce IgM and to undergo isotype switching to IgG and IgA, as well as in ascertaining the relative roles of CD4⁺ T cell stimulatory cytokines in this process.

We initially examined Ig secretion produced by sort-purified tonsil naive B cells cocultured with CXCR5⁺CD57⁻, CXCR5⁺CD57⁺, or CXCR5⁻CD57⁺ CD4⁺ T cell subsets. Both subsets of activated, but not resting, CXCR5⁺ Tfh cells induced naive B cells to secrete IgM and low levels of IgG and IgA (Fig. 6b). In contrast, CD4⁺ CXCR5⁻ T cells failed to induce secretion of any Ig isotype (Fig. 6b). Naive B cells predominantly secreted...
IgG1, followed by IgG2 and IgG3, when cocultured with PHA/IL-2-stimulated CD57+ and CD57+ T FH cells (Table II). Not surprisingly, CXCR5+ CD4+ T cells had little effect on IgG subclasses irrespective of activation (Table II).

TFH cells are a predominant source of IL-21 (70). On the other hand, there are conflicting reports as to whether human T FH cells produce IL-4 and IL-10 (66–68, 71). Because of this controversy, we examined expression of IL-4, IL-10, and IL-21 by tonsil CD4+ T cell subsets and the effect of neutralizing cytokines on the ability of T FH cells to induce isotype switching by naive B cells and their differentiation into Ig-secreting cells. Semiquantitative PCR analysis revealed expression of IL-4 by all subsets of tonsil CD4+ T cells (Fig. 6c). In contrast, IL-10 and IL-21 were predominantly expressed by CXCR5+ CD57− and CXCR5+ CD57+ T FH cells, while CXCR5+ CD57+ CD4+ T cells largely lacked expression of both of these cytokines (Fig. 6c). These data for IL-4 and IL-10 confirm those reported previously by Kim and colleagues, who examined expression and production of these cytokines by these same subsets of human tonsil CD4+ T cells (66). The greater expression of IL-10 and IL-21 by CXCR5+CD57− cells compared with CXCR5+CD57+ T FH cells (Fig. 6c) may explain the increased ability of CXCR5+ CD57− cells to induce Ig secretion by naive B cells relative to CXCR5+CD57+ T FH cells (Fig. 6b; Ref. 49). Similarly, although IL-4 was detected in CXCR5+ CD57− CD4+ T cells, this is clearly insufficient to induce naive B cell differentiation, consistent with the poor induction of Ig secretion by naive B cells cultured with CD40L and IL-4 (Fig. 3 and 4i).

When naive B cells were cocultured with CD57− or CD57+CXCR5+ T FH cells in the presence of neutralizing IL-21R-Fc, secretion of IgM and IgA was decreased 4–10-fold (Fig. 6, d and e), confirming the predominant role of IL-21 in T-dependent B cell differentiation (49, 69). IgG could not be measured in the presence of IL-21R-Fc because the human IgG Fc portion of the fusion protein cross-reacts in the IgG ELISA. In contrast to IL-21 blockade, secretion of IgM and IgA induced by CXCR5+ T FH cells was actually augmented 3–5-fold by neutralizing IL-4 (Fig. 6, d and e). This is consistent with our observations that IL-4 inhibits IL-21-induced switching to and secretion of IgA (Fig. 4), as well as secretion of IgM (49). IgG production was largely unaffected by neutralizing IL-4 (Fig. 6f).

On the other hand, while neutralizing IL-10 had no effect on CD4+CXCR5+ T cell-mediated induction of IgM (Fig. 6d), it did reduce production of IgA (Fig. 6e) and IgG (Fig. 6f), albeit not to the same extent as neutralizing IL-21. Overall, these results establish that IL-21 produced by CXCR5+ T FH cells guides the differentiation of human naive B cells into isotype-switched Ig-secreting cells, and they confirm the inhibitory effect of IL-4 on IL-21-induced secretion of IgM and IgA.

### Discussion

Ig isotype switching is a critical component of the process of B cell differentiation and the generation of protective humoral immune responses. This is most evident from the heightened susceptibility to recurrent, opportunistic, and often life-threatening infections in individuals whose B cells are unable to produce isotype-switched Ig due to mutations in genes involved in this process (e.g., CD40L, CD40, AICDA, UNG, SH2D1A, ICOS) (34). During the past two decades, cytokine-mediated regulation of isotype switching by human B cells to IgG and IgA has been extensively examined by assessing induction of Ig H chain GLT, expression of switch circles, and/or expression or secretion of IgG1, IgG2, IgG3, IgA1, or IgA2. Despite many studies, there is a great deal of controversy regarding the ability of specific cytokines to instruct B cells to produce different Ig isotypes. Some reports demonstrated that IL-4 can induce expression of γH, γ1, and γ4, but not γ2, GLTs (53, 54), while others showed that CD40L with or without IL-4 induced expression and production of all IgG subclasses (20, 55, 59, 62, 72). Similarly, while IL-4 predominantly induced secretion of IgG4 by human B cells in some studies (10, 57, 58, 73), others found that IgG1 was produced in the greatest quantities under comparable culture conditions (54, 59, 72, 74). IL-4 has even been reported to have only a small effect on IgG secretion over that observed with anti-CD40 mAb alone (19, 21, 42). There is an equal amount of uncertainty with respect to switching to IgA. While TGF-β has consistently been found to have a role in inducing human B cells to produce IgA, it is unclear whether B cell-derived or exogenous TGF-β is sufficient for this process (16–18).

On the other hand, combinations of IL-10 and TGF-β, in the absence or presence of in vitro-derived activated DC, have also been reported to be required for IgA production by human B cells (15–17), and the effect of DC is likely mediated by production of BAFF and/or APRIL (22, 23).

Some of these controversies likely derive from the use in many studies of unfractionated B cells, which contain naive and memory cells. Since memory B cells secrete much higher levels of Ig than do naive B cells, and include cells that acquired expression of IgG and IgA in vivo (61), it is difficult to discern the effects of different cytokines on bona fide isotype switching in vitro vs Ig secretion by isotype-committed cells. Furthermore, even in studies that isolated putative naive B cells by selecting IgM− or IgD− cells, the resulting population would contain IgM memory B cells (61). Since the ability of IgM memory B cells to undergo isotype switching remains unexplored, their overall contribution to Ig secretion in cultures of IgM+/IgD+ “naïve” B cells is unclear. Similarly, several studies have examined Ig isotype switching by using unfractionated B cells.
cells (13, 16, 40, 53, 62, 75), B cell clones (76, 77), immortalized B cell lines (18, 55, 62), different sources of CD40 agonists (e.g., soluble anti-CD40 mAb (10, 54, 59, 62, 76), anti-CD40 mAb presented in FcR-expressing transfectants (14, 15), recombinant CD40L (40, 42), CD40L-expressing transfectants (17, 18, 55), CD4+ T cell clones (10, 75, 77) and positively (15, 18, 21, 54, 55, 59) or negatively selected (20, 42) populations of naive B cells. Such differences in methodology do not contribute to some of the uncertainties relating to the study of Ig isotype switching by human B cells. To try to resolve some of these discrepancies, we have compared the ability of IL-4 and IL-21 to induce isotype switching and Ig secretion by human B cells. For these experiments, we cultured CFSE-labeled naive CB, PB, and splenic B cells, and IgM memory B cells, with T-dependent stimuli to analyze Ig isotype switching by subsets of mature B cells that differ with respect to Ag exposure, proliferation, and anatomical location.

We found that while IL-4 or IL-21 induced naive B cells to switch to IgG (Fig. 1), they differed with respect to the distribution of IgG subclasses and induction of IgG secretion. IL-4 predominantly directed CD40L-stimulated naive B cells to express IgG1, with fewer cells acquiring IgG2 or IgG3. Despite this, IL-4 did not induce secretion of any IgG subclasses (Fig. 2). These findings are consistent with previous studies (19, 42, 59) and reveal a dissociation between acquisition of expression of switched isotypes and the secretion of such isotypes. IL-21, on the other hand, induced IgG3 on CB B cells, IgG3 and IgG1 on naive splenic and PB B cells, and secretion of large amounts of the corresponding IgG subclass, that is, mostly IgG3 from CB B cells and similar IgG1 and IgG3 levels from adult B cells (Fig. 2). It is possible that CD40L-stimulated human B cells secrete much greater quantities of Ig in response to IL-21 than to IL-4 because IL-21 induced the autocrine production of IL-10, which potently enhances B cell differentiation (4, 14, 15) and contributes to the basal proliferation and differentiation of activated B cells (18, 63–65). However, this is unlikely because IL-10 failed to increase secretion of IgM and IgG induced by IL-21. Furthermore, while there was an effect of IL-10 on IL-21-induced production of IgA, this was only noted at concentrations of IL-10 that greatly exceeded those produced in vitro by stimulated B cells. Similarly, while the combination of IL-4 and IL-21 also induced substantial production of IL-10, IL-4 actively inhibited IgA secretion induced by CD40L/IL-21, thus making it unlikely that endogenous production of IL-10 contributes to the differentiative effects of IL-21. Since IL-6 can increase Ig production by activated human B cells and plasma cells in vitro (52, 65), it may have been anticipated that IL-4 could induce Ig secretion via the action of endogenously produced IL-6. However, CB B cells stimulated with CD40L and IL-4 failed to secrete Ig in vitro, further arguing against the likelihood that increased production of endogenous cytokines by IL-21 underlies its ability to induce secretion of substantially higher amounts of Ig than IL-4. A more likely explanation for differential Ig secretion by cytokine-stimulated B cells would be the ability of IL-21, but not IL-4, to induce expression of Blimp-1 in CD40L-stimulated naive B cells (49). Our results resemble those reported previously (19, 35); however, there are some differences that deserve comment. First, Pene et al. reported that IL-21 induced production of both IgG1 and IgG3 by anti-CD40 mAb-stimulated CD19+CD27+ splenic B cells, with the levels of IgG1 exceeding those of IgG3 by 3–10-fold (19). These differences may reflect the use of CD40L vs anti-CD40 mAb. Second, Ettinger et al. (35) found comparable secretion of IgG1 and IgG3 by IL-21-treated CD27- PB B cells; however, since these cells were also cultured with anti-CD40, anti-IgM, and IL-2, the relative roles of these stimuli in inducing IgG1/G3 were unclear. By culturing naive B cells with only CD40L and IL-21, we could demonstrate a specific effect of IL-21 on switching to IgG3 and IgG1 without the need for BCR-mediated stimulation. Our findings also highlight functional differences between naive B cells in adults and those in CB, inasmuch that most IgG produced by CD40L/IL-21-stimulated CB B cells was IgG3, while that produced by adult naive B cells was IgG1 and IgG3. Similarly, while IL-4 enhanced IgG secretion by CB B cells induced by IL-21, it had a variable effect on adult naive B cells (data not shown). Differences were also noted between naive and IgM memory B cells, where the latter expressed and produced a comparable mix of IgG1 and IgG3, as well as substantially more IgG2, in response to IL-21 (with/without IL-4) than naive B cells (Fig. 3). Thus, IgM memory B cells have more flexibility than do naive cells in the IgG subclasses that they are capable of producing following stimulation with IL-21.

Although IL-21 induced a greater proportion of naive B cells to undergo switching to express IgG than IL-4, when the effect of the combination of these cytokines was examined, IL-4 was dominant. Thus, while IgG3+ B cells were still generated, stimulation with CD40L/IL-4/IL-21 resulted in a sharp increase in IgG1+ B cells, thereby accounting for the increased proportion of IgG+ cells observed in these cultures at the bulk population (Figs. 1–3). IL-21 also induced naive B cells to switch to IgA, with IgA1 accounting for most of this response. It has been recently reported that APRIL, in combination with IL-10, induces expression of surface IgA2 by naive B cells in vitro, and these stimulated B cells can produce significant amounts of IgA2 in the additional presence of anti-Ig (23). Thus, it will be interesting to determine the combined effects of APRIL and IL-21 on expression and secretion of IgA1 and IgA2 by human B cells. In contrast to the effect of IL-4 on the generation of IgG+ cells, IL-4 dramatically attenuated IL-21-induced switching to IgA (Fig. 4). Interestingly, IL-10 could increase secretion of IgA1 induced by IL-21 (Fig. 4), consistent with a role for IL-10 in regulating IgA responses (15, 18). The physiological regulatory roles of IL-4, IL-10, and IL-21 on Ig production were confirmed from cocultures of naive B cells and CXCR5+ Tfh cells. The collaboration between IL-4 and IL-21 in regulating secretion of IgG subclasses, and the dominant effect of IL-4 on this event, was evident in these cultures inasmuch that naive B cells produced IgG1 and lower but detectable levels of IgG2 and IgG3 (Table II). This is consistent with the expression (Fig. 6) and production (66) of both IL-4 and IL-21 by CD4+ CXCR5+ Tfh cells and the skewing toward production of IgG1 by the combination of IL-4 and IL-21, compared with the predominant production of IgG3 induced by IL-21 alone (Fig. 2). While the ability of T cells to provide help for naive B cell differentiation was largely mediated by the production of IL-21 by Tfh cells, secretion of IgM and IgA was augmented by neutralizing endogenous IL-4, while neutralizing IL-10 only reduced secretion of IgG and IgA induced by Tfh cells (Fig. 6), consistent with IL-10 being a switch factor for these Ig isotypes (14, 15, 18). These studies revealed a dual regulatory role for IL-4 in modulating IL-21-induced switching by human naive B cells in an Ig isotype-specific manner. This is reminiscent of TGF-β, which can skew switching by naive B cells to IgA at the expense of IgG (17). IL-4 has previously been found to inhibit IgA secretion by total human B cells cocultured with CD4+ T cell clones (75). Furthermore, IgA secretion induced by CD4+ T cell clones was enhanced by addition of neutralizing anti-IL-4 mAb (75). This raises the possibility that the CD4+ T cell clones used in these studies were inducing IgA secretion via the production of IL-21, and that this was being antagonized by endogenous IL-4, akin to our findings. It is likely that the relative concentrations of these
cytokines produced in response to inflammatory or infectious stimuli will determine the overall quality of the humoral response with respect to the Ig isotypes produced.

A key event in isotype switching is the induction of expression of Ig H chain GLTs (2, 5, 8). The ability of cytokines to direct switching by human B cells to specific Ig isotypes and subclasses has often been correlated with their ability to induce the corresponding Ig GLT (13, 16, 18–20, 53–55, 59, 60, 76). Thus, our finding that IL-21 alone did not induce expression of IgG1 or IgG3 GLTs (Fig. 5) would appear to be inconsistent with the proposal that IL-21 can induce isotype switching to IgG1 and IgG3. However, consistent with several other studies (23, 40, 55, 62), we found that stimulation of human B cells with CD40L alone was sufficient to induce expression of most Ig H chain GLTs (Fig. 5). Based on this, we conclude that IL-21 facilitates Ig isotype switching by enhancing the proliferation of naive B cells that either acquire expression of GLTs following stimulation with CD40L alone (23, 40, 55, 62) or those that constitutively express Ig GLTs in the absence of any stimulation (53, 54, 59, 60).

Furthermore, we propose that assessment of de novo expression of downstream Ig isotypes on in vitro-stimulated naive B cells is a more accurate determinant of Ig isotype switching, since expression of Ig GLTs does not necessarily correlate with secretion or switching of Ig molecules. For instance, although CD40L induces expression of most, if not all, Ig H chain GLTs in activated B cells, these cells fail to secrete IgG1, IgG2, IgG3, IgG4, and IgA without the provision of a cytokine such as IL-4, IL-10 (14, 21, 23, 59), or IL-21 (19, 35) (Figs. 2f–h and 4i–t). Similarly, Gauchat et al. (76) found that only ~30% of B cell clones that expressed IgG GLT also secreted IgE, while Cerutti and colleagues noted that while CD40L, BAFF, and APRIL can induce GLTs in human naive B cells, secretion of IgG and IgA only occurred in cultures further supplemented with anti-Ig, IL-4, or IL-10 (22, 23, 55).

Our in vitro findings shed light on the potential mechanisms controlling the production of specific Ig subclasses under basal, infectious, and immunodeficient states. First, IgG1 represents the predominant IgG subclass in human serum, comprising ~65% of serum IgG with IgG2, IgG3, and IgG4 comprising ~25%, 10%, and 5%, respectively (1, 3). Similarly, within the population of circulating IgG+ B cells, the frequencies expressing IgG1, IgG2, IgG3, and IgG4 are ~65%, 20%, 10%, and <5% (V. L. Bryant and S. G. Tangey, unpublished data). Since the combination of IL-4 and IL-21 induced the greatest frequency of IgG1+ cells from CD40L-stimulated naive B cells (Fig. 1), it is possible that these cytokines cooperate in vivo to maintain the relatively high levels of serum IgG1 in adults. Interestingly, the distribution of IgG subclasses in serum of neonates differs from that of adults, with IgG1 and IgG3 predominating (3). Thus, it is possible that IL-21 alone has an important role in establishing serum IgG levels in neonates. This would be consistent with reduced production of IL-4 by neonatal, compared with adult, CD4+ T cells (78). Second, it is well established that IgG1 and IgG3 are elicited following acute viral infections (1–3). Despite this, the mechanism underlying the preferential generation of these subclasses during antiviral responses are unknown. Since stimulation of plasmacytoid DCs with viruses induces production of high levels of IFN-α/β (79), which in turn induces human T cells to produce IL-21 (80), it is possible that IL-21–producing CD4+ T cells are generated during viral infections following activation of plasmacytoid DCs. These CD4+ T cells could then stimulate naive B cells to produce antiviral IgG1 and IgG3 Ab. A scenario such as this would explain the predominance of serum IgG1 and IgG3 in the initial phase of antiviral immune responses. Third, the kinetics of the appearance of Ag-specific IgG subclasses during an immune response suggest specific temporal roles for cytokines that regulate switching to different isotypes and subclasses. During acute infections, virus-specific IgM, IgG1, and IgG3 Abs rapidly appear and predominate the primary humoral response. On the other hand, titers of virus-specific IgG3 decline while those of IgG1 persist and IgG4 increase either following repeated infection or in immune individuals several years after the initial infection (24–27). These observations suggest a dominant role for IL-21 in primary antiviral responses, but a role for IL-21 together with IL-4 in the maintenance of long-term humoral immunity. The detection of virus-specific IgM and IgA1 only during acute rubella infection (26), coupled with our finding that IL-4 suppresses secretion of IgM and IgA induced by CD40L/IL-21 in vitro (Fig. 4) (49), also supports a role for IL-21 in the initiation, yet IL-21 plus IL-4 in the maintenance, of antiviral humoral immune responses. Lastly, since IL-21 can induce adult naive B cells to switch to IgG1 and IgG3 (Fig. 2) (19), and IgG3 deficiency is often associated with an IgG1 deficiency (3), it is possible that defective production of IL-21 from CD4+ T cells underlies the development of such IgG subclass immunodeficiencies. Similarly, the finding that IL-21 can induce switching to IgG and IgA suggests that the severe deficit in serum Ig in patients with X-SCID, due to mutations in γc (56), most likely results from an inability to receive stimulatory signals through the IL-21R/γc complex, rather than other γc-containing cytokine receptors, such as those for IL-2 or IL-4. In summary, our findings have revealed novel roles for IL-4– and IL-21 in regulating the expression and production of IgG and IgA subclasses by naive human B cells. Developing strategies by which the activity or availability of these regulatory cytokines are altered, and determining the signals and conditions required for the production of IL-21 by Tfh cells, may provide avenues by which humoral immune responses in normal and disease settings could be beneficially modulated.

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Disclosures
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