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IL-21 Induces Differentiation of Human Naive and Memory B Cells into Antibody-Secreting Plasma Cells

Rachel Ettinger,* Gary P. Sims,* Anna-Marie Fairhurst,* Rachel Robbins,* Yong Sing da Silva,* Rosanne Spolski,† Warren J. Leonard,† and Peter E. Lipsky*

IL-21 is a type I cytokine that influences the function of T cells, NK cells, and B cells. In this study, we report that IL-21 plays a major role in stimulating the differentiation of human B cells. When human B cells were stimulated through the BCR, IL-21 induced minimal proliferation, IgD down-modulation, and small numbers of plasma cells. In contrast, after CD40 engagement, IL-21 induced extensive proliferation, class switch recombination (CSR), and plasma cell differentiation. Upon cross-linking both BCR and CD40, IL-21 induced the largest numbers of plasma cells. IL-21 drove both postswitch memory cells as well as poorly responsive naive cord blood B cells to differentiate into plasma cells. The effect of IL-21 was more potent than the combination of IL-2 and IL-10, especially when responsiveness of cord blood B cells was examined. IL-21 costimulation potently induced the expression of both B lymphocyte-induced maturation protein-1 (BLIMP-1) and activation-induced cytidine deaminase as well as the production of large amounts of IgG from B cells. Despite the induction of activation-induced cytidine deaminase and CSR, IL-21 did not induce somatic hypermutation. Finally, IL-2 enhanced the effects of IL-21, whereas IL-4 inhibited IL-21-induced plasma cell differentiation. Taken together, our data show that IL-21 plays a central role in CSR and plasma cell differentiation during T cell-dependent B cell responses. The Journal of Immunology, 2005, 176: 7867–7879.

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1 This work was supported in part by the Intramural Research Program of the National Institutes of Health, National Institute of Arthritis and Musculoskeletal and Skin Diseases. The specific signals that drive the differentiation of human naive B cells into memory and plasma cells has not been as extensively characterized as that of their murine counterparts. However, a variety of studies have suggested that cytokines, such as IL-2, IL-6, and IL-10 are involved in human plasma cell differentiation (11–21). Moreover, in vitro and in vivo analyses have identified cellular interactions, including those mediated by CD40 and its ligand, CD154, as playing pivotal roles in the generation of both memory B cells and plasma cells (22–24). Nonetheless, responsiveness of B cells and particularly naive B cells is minimal in the presence of purified costimulators (21, 25–28). The specific signals that drive the differentiation of human naive B cells into memory cells and plasma cells, therefore, have not been completely defined, and an integrated view of these essential steps in human B cell biology has not been definitively established.

Because IL-21 plays an important role in murine B cell differentiation into memory cells and plasma cells, we investigated the role of IL-21 in human B cell differentiation. In this study, we report that IL-21 costimulation not only is capable of inducing plasma cell differentiation from CD27+ memory B cells, but also has the capacity to induce class switch recombination (CSR) and stimulate poorly responsive naive cord blood B cells into IgG-secreting plasma cells. Importantly, IL-21 costimulation up-regulated expression of both activation-induced cytokine deaminase (AID) and B lymphocyte-induced maturation protein-1 (BLIMP-1), but did not induce somatic hypermutation (SHM). Finally, the action of IL-21 was largely prevented by another type I cytokine, IL-4. These results demonstrate that IL-21 is one of the major T cell influences that initiates CSR and differentiation of human B cells into Ab-secreting plasma cells. Moreover, the interplay of IL-21 and IL-4 may exert an essential function in determining the outcome of human humoral immune responses.

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† Abbreviations used in this paper: γc, common cytokine receptor γ-chain; CSR, class switch recombination; AID, activation-induced cytidine deaminase; BLIMP-1, B lymphocyte-induced maturation protein-1; SHM, somatic hypermutation; PB, peripheral blood; SAC, Staphylococcus aureus Cowan I; PI, propidium iodide; HU, hydroxyurea; β2M, β2 microglobulin; int, intermediate.

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Materials and Methods

Isolation of human B cells

All human studies have been approved by the Warren G. Magnuson Clinical Center Institutional Review Board, and informed consent was obtained according to the declaration of Helsinki. Human peripheral B cells were isolated fromuffy coats of anonymous healthy donors drawn at the National Institutes of Health Division of Transfusion Medicine. Umbilical cord blood was harvested after delivery and either harvested from ACD (R&D Systems) or 5 × 10^6 cells/ml in either 1 ml in 24-well culture plates or 100 μl in 96-well bottom-round bottom culture plates. The cells were incubated with a combination of human IL-2 (100 U/ml; Roche), human IL-4 (100 ng/ml; R&D Systems), human IL-21 (100 ng/ml; R&D Systems) and either 1 μg/ml anti-human CD40 (R&D Systems), 5 μg/ml anti-IgM (Jackson ImmunoResearch Laboratories), or 0.01% final dilution of stimulation. Wnt to 100 cells/ml in staining buffer with mAbs specific for CD20 and CD27 and in some experiments anti-IgD as well. Cells were incubated for 30 min at 4°C, washed, and sorted into CD20^−CD27^− or CD20^+CD27^ memory B cells were isolated on a MoFlo cell sorter (DakoCytomation). Briefly, negatively selected B cells were incubated at 10^6 cells/ml in staining buffer with mAbs specific for CD20 and CD27 and in some experiments anti-IgD as well. Cells were incubated for 30 min at 4°C, washed, and sorted into CD20^−CD27^− or CD20^+CD27^ memory B cells. Preparations were typically >98% pure.

Cell culture, activation, and CFSE labeling

Purified B cells were cultured at 1 × 10^6 cells/ml in either 1 ml in 24-well culture plates or 100 μl in 96-well bottom-round bottom culture plates. The cells were incubated with a combination of human IL-2 (100 U/ml; Roche), human IL-4 (100 ng/ml; R&D Systems), human IL-21 (100 ng/ml; R&D Systems) and either 1 μg/ml anti-human CD40 (R&D Systems), 5 μg/ml anti-IgM (Jackson ImmunoResearch Laboratories), or 0.01% final dilution of heat-killed, formalin-fixed Staphylococcus aureus Cowan I (SAC) (Calbiochem). When multiple cytokines and stimuli were used, all cytokines and stimuli were added at the initiation of culture. In some experiments, purified B cells were labeled with CFSE. In brief, purified B cells were washed extensively in PBS to remove all FCS, and CFSE/PBS (Molecular Biochemicals) was added to the cell suspension and the cells were typically >96% pure. For further purity of peripheral B cells, CD20^−CD27^ naive B cells or CD20^+CD27^ memory B cells were isolated on a MoFlo cell sorter (DakoCytomation). Briefly, negatively selected B cells were incubated at 10^6 cells/ml in staining buffer with mAbs specific for CD20 and CD27 and in some experiments anti-IgD as well. Cells were incubated for 30 min at 4°C, washed, and sorted into CD20^−CD27^− or CD20^+CD27^ subsets. Preparations were typically >98% pure.

Flow cytometry

Four-color flow cytometry was performed using a FACSCalibur (BD Biosciences). Briefly, supernatants were collected, and then all cells were harvested from 96-well cultures at the end of the incubation period and stained for 30 min on ice with a combination of mAbs (BD Biosciences). The combination of anti-IgD-FITC, anti-CD19-PerCP-Cy5.5, and anti-CD38-APC (Bender MedSystems) and B cell maturation Ag (BCMA)-biotin (R&D Systems) followed by streptavidin-PE. In addition, the combination of anti-CD31-FITC, anti-IgD-PE, anti-CD19-PerCP-Cy5.5, and anti-CD38-APC, or anti-IgD-FITC, anti-CD19-PerCP-Cy5.5, and anti-CD38-APC were also used (all obtained from BD Biosciences), or IL-6R-biotin (Bender MedSystems) and B cell maturation Ag (BCMA)-biotin (R&D Systems) followed by streptavidin-PE. To assess proliferative responses of cultured cells, 10^6 purified B cells were cultured as described above in 96-well round-bottom plates. After 3–5 days of culture, [3H]thymidine (37 Kbq/well) was added to the cultures for an additional 16 h. Thymidine uptake was measured using a liquid scintillation counter. To assess whether IL-21-induced plasma cells were in cell cycle, two strategies were used. First, propidium iodide (PI) incorporation was used to determine the cell cycle position of the cells. Stimulated and control cultures were stained after various days with either anti-IgD-FITC, anti-CD19 PerCP-Cy5.5, and anti-CD38-APC to visualize plasma cells based on IgD and CD38 expression or anti-CD27-FITC and anti-CD38-APC, followed by 1% paraformaldehyde solution for 15 min, followed by the addition of 100 μg/ml PI solution in PBS with 0.1% Triton X-100 for at least 1 h before analyses. Second, hydroxyurea (HU) (Sigma Chemicals) was added to some cultures at a final concentration of 10^−3 M at either day 0 (that were subsequently either pulsed with [3H]thymidine after 3 days or stained for flow cytometric analysis or analyzed for IgG production after 8 days) or at 8 days of culture (that were subsequently either stained for flow cytometry or analyzed for IgG production at 11 days of culture). This combination of HU has been shown to completely inhibit the proliferation of activated cycling human B cells (17).

Real-time quantitative PCR

Negatively or positively purified B cells were stimulated as described above. After 3 days in culture, cells were harvested and resuspended in TRIzol (Invitrogen Life Technologies) and stored at −70°C. RNA was isolated using the RNeasy mini kit (Qiagen). Purity was measured using spectrophotometry. Reverse transcription reactions were prepared using the SuperScript One-Step PCR System with Platinum Taq Polymerase and ROX reference dye (Invitrogen Life Technologies). Fifty nanograms of isolated RNA was added per reaction with 1.2 mM MgSO4, TaqMan MxPays-on Demand Gene expression primer/probe sets (Applied Biosystems) were used for BCLIP-1 (Hs00153357_ml), Bcl6 (Hs00153368_ml), AICDA (Hs00221068_ml), PAX-5 (Hs00277134_ml), and B2MIC globalulin (Hs00999907_ml). Final concentrations were 1.8 μM for primers and 0.5 μM for probes. RT-PCR was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems), and cycle conditions and relative quantification were completed as described by manufacturer’s instructions (Applied Biosystems). Expression of each transcription factor was calculated using the comparative computerized tomography method with efficiency calculations and with all mRNA levels absorbance was measured, and OD was quantified at 410 nM by a PowerWave X 96-well plate reader (Bio-Tek Instruments). IgE concentrations were measured with the Human IgE ELISA kit (Bethyl Laboratories). Specific IgG isotypes were differentiated using the Human IgG Subclass Profile ELISA KIT (Zymed Laboratories).

B cell proliferation and cell cycling

To assess proliferative responses of cultured cells, 10^6 purified B cells were cultured as described above in 96-well round-bottom plates. After 3–5 days of culture, [3H]thymidine (37 Kbq/well) was added to the cultures for an additional 16 h. Thymidine uptake was measured using a liquid scintillation counter. To assess whether IL-21-induced plasma cells were in cell cycle, two strategies were used. First, propidium iodide (PI) incorporation was used to determine the cell cycle position of the cells. Stimulated and control cultures were stained after various days with either anti-IgD-FITC, anti-CD19 PerCP-Cy5.5, and anti-CD38-APC to visualize plasma cells based on IgD and CD38 expression or anti-CD27-FITC and anti-CD38-APC, followed by 1% paraformaldehyde solution for 15 min, followed by the addition of 100 μg/ml PI solution in PBS with 0.1% Triton X-100 for at least 1 h before analyses. Second, hydroxyurea (HU) (Sigma Chemicals) was added to some cultures at a final concentration of 10^−3 M at either day 0 (that were subsequently either pulsed with [3H]thymidine after 3 days or stained for flow cytometric analysis or analyzed for IgG production after 8 days) or at 8 days of culture (that were subsequently either stained for flow cytometry or analyzed for IgG production at 11 days of culture). This combination of HU has been shown to completely inhibit the proliferation of activated cycling human B cells (17).

FIGURE 1. IL-21 induces plasma cell differentiation after stimulation with anti-CD40 and anti-IgM. Purified PB B cells were stimulated with no polyclonal B cell activator or with the combination of anti-CD40 and anti-IgM in the presence or absence of IL-21 as indicated. After 6 days of culture, B cells were identified based on CD19 expression and assessed for expression of IgD and CD38. IgD^−CD38^high plasma cells are present in the lower right quadrant of anti-CD40, anti-IgM, and IL-21-stimulated cultures. The numbers in the quadrants indicate the percentage of CD19^+ cells in each region. Cell surface phenotype of B cells before culture is also shown (day 0).
normalized to β_{2}M. All reported values were then further normalized to control conditions, of cultures from PB B cells with no cytokine and no stimuli or cultures from cord blood B cells with IL-2 (Biological Research Branch, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Frederick, Maryland) and IL-4 (R&D Systems) without any stimuli, as a value of 1.

**Somatic hypermutation**

B cells enriched from four cord blood samples were stimulated with IL-2 and IL-21 in the presence of anti-CD40 or anti-CD40 and anti-IgM for 7–12 days. Individual live, CD19^{+} IgD^{−} CD38^{high} plasma cells were sorted, and the rearranged Ig H chain V regions (V_{H}) from genomic DNA of single cells was amplified and directly sequenced as described previously (29). The V_{H} gene sequences from these IL-21-induced plasma cells, and unstimulated cord blood B cells collected immediately after purification, were compared with germline genes and their polymorphic variants from our own and public databases used to determine the closest germline gene and the number of V_{H} gene segment mismatches. Several undocumented but recurring V_{H} gene mismatches that we have found in genes isolated from naive B cells in multiple individuals were considered putative polymorphic variants and were excluded from the analysis. Data obtained from plasma cells derived from anti-CD40 and anti-CD40 and anti-IgM-stimulated cultures were similar, and their data were pooled for the analysis.

**Results**

**IL-21 induces plasma cell differentiation**

The capacity of IL-21 to costimulate responses of purified human B cells was initially explored. Freshly obtained B cells or those retrieved from cultures were stained with anti-IgD, anti-IgM, anti-CD40, both stimuli, or neither in the presence or absence of IL-2 and/or IL-21 as indicated. B. After 3 days of culture, proliferation was determined by incubating the cells for 16 h with [³H]thymidine. C. After 6 days of culture, the cells were stained and B cells identified based on CD19 and CD38 expression as shown in Fig. 1, and analyzed for surface expression of IgD and CD38. Results from a representative experiment of six similar experiments are shown. D. Purified B cells were first labeled with CFSE before being cultured. After 7 days of culture, CD19^{+} B cells were analyzed for CD38 expression and CFSE dilution. Data are representative of results from three similar experiments.
resulted in marked down-modulation of IgD and substantial differentiation of plasma cells that were phenotypically identified as CD19<sup>high</sup>/IgD<sup>low</sup> cells (Fig. 1). The next experiments examined the impact of IL-21 in detail and compared it with another type I cytokine that has been reported to support Ig production from human B cells, IL-2 (30). As shown in Fig. 2A, only a small fraction (1.3%) of freshly isolated peripheral B cells are IgD<sup>−</sup>CD38<sup>high</sup> plasma cells; the majority are IgD<sup>−</sup>CD38<sup>low</sup> naive B cells, and a smaller percentage are IgD<sup>−</sup>CD38<sup>−</sup> postswitched memory B cells. In the presence of anti-CD40 (which can stimulate both naïve and memory B cells), IL-21 induced maximal proliferation of B cells, whereas IL-2 had little effect (Fig. 2B). In contrast, in the presence of anti-IgM (which stimulates IgD<sup>+</sup>/IgM<sup>+</sup> B cells only), IL-21 induced only minimal proliferation. Notably, IL-2 enhanced the proliferation of B cells stimulated with anti-IgM and IL-21, whereas it had little effect on B cells stimulated with anti-CD40 and IL-21. When B cells were triggered through both CD40 and IgM, IL-21 induced a proliferative response that was comparable to that noted with anti-CD40 and IL-21. As with the anti-IgM and IL-21 costimulation, IL-2 also increased the magnitude of the response when cells were stimulated with the combination of IL-21, anti-IgM, and anti-CD40.

Flow cytometric evaluation was conducted to assess the impact of IL-21 on human B cells in greater detail. When negatively selected peripheral B cells were cultured with cytokines alone, modest changes in B cell phenotype were noted as determined by IgD and CD38 expression (Fig. 2C, a–d). It was notable, however, that IL-21 in the absence of any other stimulus increased the percentage of IgD<sup>−</sup>CD38<sup>high</sup> plasma cells modestly, and this effect was augmented by IL-2 (Fig. 2C, c and d). The ability of IL-21 to induce moderate plasma cell differentiation was also observed with anti-IgM-stimulated B cells, and again there was an increase in the presence of IL-2 (Fig. 2C, g and h). In addition, IL-21 induced a striking loss in the numbers of B cells in anti-IgM-stimulated cultures (Fig. 2C, compare g to e), which was largely reversed by the addition of IL-2 (Fig. 2C, compare h to g). Moreover, IL-21 or the combination of IL-2 and IL-21 induced marked down-regulation of surface IgD by anti-IgM-stimulated B cells (Fig. 2C, g and h). IL-21 also induced IgD down-modulation by anti-CD40-stimulated B cells, as well as a dramatic increase in cellular expansion and plasma cell differentiation that was observed as early as day 4 of culture (Fig. 2C, compare k to i and j and s). T cell-dependent B cell responses involve engagement of both surface Ig as well as CD40 by CD154 expressed by activated T cells. It was therefore of interest to examine the impact of IL-21 on B cells stimulated through both the BCR and CD40. We found that the combination of anti-IgM and anti-CD40 in the presence of IL-21 resulted in down-modulation of IgD by nearly all B cells (Fig. 2C, compare o to m). Moreover, the largest percentage of plasma cells was generated when both surface IgM and CD40 were engaged, and the cells were costimulated with IL-21 in the presence or absence of IL-2 (Fig. 2C, o and p). Importantly, evaluation of CFSE dilution revealed that IL-21 induced the differentiation of plasma cells from a dividing B cell precursor that had diluted CFSE with or without costimulation (Fig. 2D). Numeric calculation in repetitive experiments demonstrated the significant increase in both the percentage (Fig. 3A) and absolute cell number (Fig. 3B) of plasma cells in cultures costimulated with IL-21.

IL-21 induces plasma cell differentiation from naive cord blood B cells
CD27 expression denotes that human B cells have somatically mutated Ig genes and, therefore, are considered to be memory B cells (31). These include both IgD<sup>−</sup>CD27<sup>+</sup> B cells as well as IgD<sup>−</sup>CD27<sup>−</sup> postswitched B cells. To address whether IL-21 had the capability to drive plasma cell differentiation from both naive and memory B cells, cord blood B cells were used as a natural source of naïve CD27<sup>−</sup> IgD<sup>−</sup> B cells, and CD27<sup>+</sup> (IgD<sup>−</sup> and IgD<sup>+</sup>) memory B cells were isolated from PB by cell sorting. As shown in Fig. 4, A and B, IL-21 costimulated considerable proliferation of both naive cord blood and memory B cells. Although cord blood B cells costimulated with IL-21 proliferated less well than adult CD27<sup>+</sup> memory B cells (compare Fig. 4, B to A), the capacity of IL-21 to down-modulate IgD and induce differentiation of plasma cells was dramatic in both populations (Fig. 4, C and D). IL-21 with anti-CD40 or anti-IgM and anti-CD40 (but not with anti-IgM alone) had the capacity to induce the generation of plasma cells from nearly all the CD27<sup>−</sup>IgD<sup>−</sup> and CD27<sup>+</sup>IgD<sup>−</sup> IgD<sup>+</sup> memory B cells (Fig. 4D). More notable was the finding that nearly all surviving IL-21-stimulated cord blood B cells activated with either anti-CD40 or anti-IgM and anti-CD40 had down-modulated IgD and most differentiated into plasma cells. It is important to note that neither anti-CD40 nor anti-IgM and anti-CD40 stimulation of cord blood B cells alone led to the generation of any plasma cells in the absence of IL-21 (Fig. 4C). However, IL-21 induced the down-modulation of IgD on stimulated cord blood B cells within 3 days of culture (data not shown), and by day 6 many plasma cells were observed (Fig. 4C). In contrast, IL-21-induced differentiation of plasma cells from adult PB B cells could be observed within 4 days of culture (data not shown).

IL-21 induces Ig secretion from both naive and memory B cells
Analysis of culture supernatants confirmed that IL-21 induced the secretion of Ig. Unlike IL-2, or cultures with no added cytokines, IL-21 costimulated considerable production of both IgG and IgM from total adult peripheral B cells, as well as naive cord blood B cells activated with anti-CD40 or anti-IgM and anti-CD40 (Fig. 5, A and B). The amount of Ig produced generally correlated with the frequency of plasma cells in the cultures. IgM and IgG could be assayed in the culture supernatants as early as day 4 of culture (data not shown). It is notable that IL-21 induced large amounts of...
FIGURE 4. IL-21 induces CSR and plasma cell differentiation from both naive and memory B cells. B cells were positively selected from cord blood, or negatively selected from PB, and the latter were further purified into CD20−CD27− memory B cells by cell sorting. All cells were cultured with anti-IgM, anti-CD40, both stimuli, or neither in the presence or absence of IL-21. B cell populations were stimulated as indicated, and proliferative responses were measured by [3H]thymidine incorporation after 3 days in culture of either cord blood B cells (A) or CD27− memory B cells (B). The purity of the cord blood and CD20−CD27− memory B cells before culture is shown in (C and D). Neither cord blood B cells nor CD20−CD27+ memory cells contained identifiable plasma cells before culture. After 6 days of culture, the cells were stained and identified based on CD19 and CD38 expression as shown in (Fig. 1), and analyzed for IgG1 and IgG3. No difference was observed in proliferation or change in phenotype of cord blood B cells isolated by negative vs positive selection. Data are representative of results from one of six experiments for purified cord blood B cells and one of five similar experiments for CD27− sorted PB B cells.

IL-21 induces the differentiation of both cycling and terminally differentiated cells

We next addressed the state of the IL-21-induced plasma cells both by cell surface phenotype and cell cycle analysis. In addition to being CD19lowIgD−CD38high, the majority of IL-21, anti-CD40, and anti-IgM-induced plasma cells (red) were also found to express other typical plasma cell markers such as IL-6R and B cell maturation Ag (BCMA) (Fig. 6). Furthermore, when compared with IgD−CD38low cells (blue), these plasma cells were CD40−, CD95low, HLA-DRlow, CD99high, CD63high, CXCR4low, CD44high, CD49dhigh, CD31high, and CD62Lhigh (Fig. 6). The majority of the IL-21-driven plasma cells were also CD19low, CD20low, CD22low, CD21low, and CD27high (data not shown).

To evaluate whether IL-21-induced plasma cells were terminally differentiated nondividing cells, cell cycle analysis was also conducted. To assess cell cycle position, B cells were stimulated with IL-21, anti-IgM, and anti-CD40 and after 7 days of culture the resultant plasma cells were identified either as IgG−CD38high (Fig. 7B) or CD27highCD38high cells that were differentiated from CD27lowCD38low nonplasma cells (Fig. 7C). Both populations were analyzed for cell cycle progression by PI staining (Fig. 7D). As shown in Fig. 7D, approximately one-third of IL-21-induced plasma cells were cycling, whereas the majority were not cycling as evidenced by the finding that they were not in the S, G2, or M phase of the cell cycle. It is notable that approximately one-fourth of the other B cells in the culture were in cell cycle after 7 days of culture, although 46% of these nonplasma cells were found to be in S/G2/M on day 3, before plasma cell differentiation (data not shown).

Finally, to address whether IL-21-induced plasma cells had differentiated to nondividing Ig-secreting cells, Ig production was examined in the presence of the inhibitor of proliferation, HU. HU is an inhibitor of proliferation that prevents Ig secretion by cycling but not noncycling Ig-secreting cells (17, 32). The addition of HU at the initiation of culture resulted in a complete block of IL-21-induced proliferation and plasma cell differentiation (Fig. 8, A and B). However, when HU was added to the cultures at day 8 after differentiation of plasma cells, persistence of these cells was noted over the next 3 days of culture (Fig. 8B). Importantly, the amount of Ig doubled during the last 3 days of culture, which was not blocked by HU, indicating that the ongoing production of Ig by plasma cells was not dependent on ongoing proliferation. These data demonstrate that the production of Ig by IL-21-induced
plasma cells largely reflects secretory activity of terminally differentiated, nondividing cells (Fig. 8C).

**IL-21 induces expression of BLIMP-1, AID, and Bcl-6 mRNA, but not SHM**

A variety of transcription factors are known to regulate specific stages of B cell maturation. It was, therefore, of interest to determine whether IL-21 costimulation would up-regulate expression of BLIMP-1, which is essential for plasma cell differentiation (33), AID, which is involved in CSR (34), Bcl-6, which is involved in germinal center reactions (35), or PAX-5, which is required for the generation of B cells (36). IL-21 induced expression of both BLIMP-1 (Fig. 9A, a) and AID (Fig. 9A, b) mRNA. BLIMP-1 was induced by IL-21-costimulated B cells activated with anti-CD40 or anti-IgM and anti-CD40, and less so with anti-IgM only (Fig. 9A, a). However, in the presence of IL-2, IL-21 also induced BLIMP-1 expression without additional costimulation. AID mRNA was induced by IL-21 when B cells were activated with anti-IgM, anti-CD40, or both anti-IgM and anti-CD40, and was enhanced by the presence of IL-2. Bcl-6 mRNA was induced to a lesser degree by IL-21 (Fig. 9A, c), whereas IL-21 had little effect on PAX-5 mRNA (Fig. 9A, d). To determine whether IL-21 had the ability to induce these transcription factors in naive B cells, experiments using cord blood B cells were undertaken. To obtain sufficient mRNA for analysis, cord blood B cells were cultured with viability promoting cytokines, IL-2 and IL-4, which in preliminary experiments did not induce the mRNAs analyzed. IL-21 (in the presence of IL-2) induced a significant increase in BLIMP-1 mRNA in naive anti-CD40 or anti-CD40 and anti-IgM-stimulated cord blood B cells, whereas the combination of IL-2 and IL-4 exhibited no such activity (Fig. 9B). IL-21 also costimulated AID mRNA expression, but no more effectively than the combination of IL-2 and IL-4 (Fig. 9B). In contrast, IL-21 had little effect on Bcl-6 or PAX-5 mRNA levels.

It was notable that IL-21 costimulation increased AID mRNA levels, differentiation of IgD+/H11001 and IgD+/H11002 B cells, CSR, generation of plasma cells, and Ig production, but it could not induce SHM. Compared with unstimulated cord blood B cells, no significant increase in the percentage of sequences with VH gene mismatches was detected in isolated IgD-CD38high plasma cells induced from cord blood B cells stimulated with IL-2 and IL-21 in the presence of anti-CD40 or anti-CD40 and anti-IgM (Fig. 9C).
percentage of sequences with mismatches most likely reflected unidentified VH gene polymorphisms or Taq-induced PCR misincorporation events, rather than bona fide somatic mutations.

**IL-4 suppresses plasma cell generation promoted by IL-21**

The effects of IL-21 can be modulated by other cytokines. As shown in Fig. 10A, IL-4 partially inhibited the down-modulation of surface IgD induced by IL-21 in cells stimulated with the polyclonal B cell activator, SAC (Fig. 10A, compare e to f). Moreover, IL-4 also inhibited IgD down-modulation induced by IL-21 and anti-IgM or the combination of anti-IgM and anti-CD40 (Fig. 10A, compare h and n to i and o, respectively), but not by anti-CD40 alone (Fig. 10A, compare j to k). Importantly, IL-21-induced generation of plasma cells was also inhibited by IL-4 in cultures co-stimulated by SAC (Fig. 10A, compare e to f), or in cultures containing the combination of IL-21, anti-IgM, and anti-CD40 (Fig. 10A, compare n to o) but not in cultures of IL-21 and anti-CD40 (Fig. 10A, compare k to i) reproducibly. This was reflected in the quantity of secreted Ig (Fig. 10B), as well as the levels of AID or BLIMP-1 mRNA (Fig. 10C). Thus, IL-4 inhibited IL-21-induced up-regulation of Ig production as well as BLIMP-1 and AID mRNAs in cultures stimulated by SAC or anti-CD40 with anti-IgM, but not reproducibly in those stimulated by anti-CD40. Notably, IL-2 did not reproducibly affect IgG production or BLIMP-1 or AID mRNA levels in these cultures. Finally, the addition of IL-2 did not rescue IgG production in IL-4-suppressed cultures (Fig. 10B), but did allow for the differentiation of IgM-producing plasma cells (data not shown).

**IL-21 induces greater plasma cell differentiation than does the combination of IL-2 and IL-10**

The next experiments compared the impact of IL-21 with that of the combination of IL-2 and IL-10 that has been shown to support plasma cell differentiation under some circumstances (13, 20). As shown in Fig. 11, stimulation of purified PB B cells with anti-CD40 and the combination of IL-2 and IL-10 induced proliferation (Fig. 11A), as well as the differentiation of plasma cells (Fig. 11B) and IgG secretion (Fig. 11C), although the responses were not as robust as those induced by anti-CD40 and IL-21. In contrast, stimulation of naive cord blood B cells with anti-CD40 and the combination of IL-2 and IL-10 failed to stimulate any of these responses, although IL-21 induced proliferation, plasma cell generation, and IgG secretion from anti-CD40-stimulated cord blood B cells (Fig. 11A–C). Notably, however, the combination of IL-2 and IL-10 induced up-regulation of both BLIMP-1 and AID mRNA, although AID mRNA was induced substantially more effectively by IL-21 (Fig. 11D).

**Discussion**

During T cell-dependent responses in a germinal center, naive B cells receive a combination of signals from encounter with Ag and T cells that result in clonal expansion, CSR, SHM, and the differentiation of memory and plasma cells. Studies in the mouse have defined a number of these specific processes and factors. Recently, IL-21 has been shown to be an essential factor in the generation of plasma cells that produce IgG (10). Less is known about the control of these processes in humans. Although a number of cytokines and T cell influences are known to regulate B cell responses, the specific factors that stimulate the differentiation of naive human B cells into plasma cells secreting IgG have not been well defined. Indeed, both adult and especially cord blood naive human B cells have been shown to have a poor propensity to differentiate into plasma cells in vitro, especially in the presence of purified co-stimulators (11, 13, 21, 25, 27). However, a modest capacity to differentiate into Ig-producing plasma cells has been reported when naive B cells are stimulated through CD40, in the presence of L cells, and supported by IL-10 (12) or activated by the combination of SAC, IL-2, IL-10, and cross-linked anti-CD40 (18). Others have found no plasma cell generation when naive CD27− B cells are stimulated with CD40L transfectants in combination with IL-2, IL-4, IL-6, IL-10, and IL-12 (21).

We now show that IL-21 is a powerful costimulator of human CSR and plasma cell differentiation, but not SHM. Importantly, IL-21 can promote both CSR and plasma cell differentiation from naive as well as memory B cells. Notably, the combination of anti-IgM and anti-CD40, which most closely mimics B cell activation via Ag and T cell interaction, was the most effective signal in promoting the maximal differentiation of plasma cells driven by IL-21. Although anti-CD40 and IL-21 induced considerable CSR and plasma cell differentiation, the “T cell only” signal was somewhat less effective than the combination. Isolated BCR cross-linking, which mimics the “Ag-only” signal, primed B cells for IL-21-mediated death (Fig. 1C, g, and our unpublished observations). These results suggest that IL-21 is pivotal in cell fate decisions by activated B cells and may function to eliminate B cells that have been activated by Ags or autoantigens in the absence of T cell signals.

IL-21 has been reported to be a switch factor for IgG1 and IgG3 (26), although in this study we found that the nature of the B cell population contributed to the specific Ig produced. Thus, IL-21-stimulated cord blood B cells predominantly switched to IgG3, whereas naive adult B cells switched to both IgG1 and IgG3. The
molecular basis for the differences in these outcomes is currently unknown, but clearly the nature of the B cell population contributes to the specificity of switch recombination. Notably, memory B cells were induced by IL-21 to produce all IgG isotypes as well as IgA. This is somewhat different from previous results with CD19-H11001 splenic B cells in which IgA was not induced by IL-21 (26). The explanation for this difference is currently unknown. This may reflect differences in peripheral and splenic B cells.

The combination of IL-2 and IL-10 has been reported to induce human plasma cell differentiation from memory, but not naive B cells (13, 20, 25). In contrast to IL-21, a variety of other cytokines, including IL-2, IL-4, IL-6, and IL-10 (data not shown), had minimal ability to support the generation of plasma cells from comparably stimulated B cells. Importantly, although the combination of IL-2, IL-10, and anti-CD40 did induce some plasma cell differentiation from PB B cells, this stimulus could not induce plasma cell differentiation from naive cord blood B cells and has been reported previously (25). These data indicate that IL-21 was a far more potent inducer of plasma cell differentiation than the combination of IL-2 and IL-10 and may be uniquely able to foster differentiation of plasma cells from naive B cells.

BLIMP-1 is a transcriptional repressor that is necessary/sufficient for plasma cell differentiation in the mouse, and in human B cell lines (33, 37, 38). Consistent with this, IL-21-driven plasma cell differentiation from both naive cord blood B cells as well as CD27+ memory B cells was preceded by the induction of BLIMP-1 up-regulation. We have also found that IL-21 induced BLIMP-1 expression in murine splenic B cells (10). The capacity of IL-21 to up-regulate BLIMP-1 may explain its ability to drive plasma cell differentiation. In addition, IL-21 costimulation induced AID expression, which is normally down-modulated by BLIMP-1 (38). Although AID is required and believed to be the B cell-specific factor that is sufficient for both CSR and SHM (34), IL-21 costimulation induced CSR, but not SHM. Similar findings have been reported with CD27-H11002 B cells stimulated with the combination of SAC, IL-2, IL-10, and cross-linked anti-CD40 (18). Of note, we found that IL-4 and CD40 ligation induced AID in cord blood B cells, but neither CSR nor plasma cell differentiation. Previously, AID has been shown to be induced by IL-4, anti-CD40, or the combination of both IL-4 and anti-CD40 in adult B cells (26, 39, 40). It is of particular interest that AID was induced comparably by IL-4 and IL-21, although only the latter induced CSR. In this regard, engagement of CD40 induced both AID and SHM in human Ramos B cell lines (41, 42). These results suggest that up-regulation of AID may have different effects on B cells at different stages of differentiation. Moreover, our data indicate that the induction of AID mRNA is not sufficient to induce CSR or SHM in all circumstances. The property of IL-21 that permits it to induce both AID and CSR is currently unknown, but of great interest. The
finding that IL-21 can induce AID and CSR but not SHM is reminiscent of the recent delineation of AID function, indicating that different portions of the molecule govern CSR and SHM. Presumably IL-21 can induce CSR, the activity of AID that is governed by the C-terminal portion of the molecule and involves the capacity to bind DNA-protein kinase catalytic subunit (43), whereas it is not able to induce SHM that is regulated by the N-terminal of the protein by unknown molecules (44). The biological basis of this functional discrimination is an important area for future investigation. Finally, the combination of IL-2, IL-10, and anti-CD40 was able to induce BLIMP-1 mRNA, but far less AID mRNA from PB B cells, suggesting that this cytokine pair may be unable to induce CSR, and may explain its inability to induce the differentiation of postswitched plasma cells from naive cord blood B cells. Moreover, the data suggest that the capacity to induce BLIMP-1 may be necessary for plasma cell differentiation, but may not be the sole determinant of the ability of IL-21 to induce the differentiation of large numbers of plasma cells from both naive and memory B cells.

It was possible that IL-21 may have functioned as a plasma cell survival factor, rather than as an initiator of plasma cell differentiation. This seems unlikely for a number of reasons. First, IL-21 alone had little capacity to maintain the few plasma cells that were found in the initial population of peripheral CD19+ cells.Secondly, in cultures costimulated with IL-21, plasma cells appeared after 3–6 days, whereas in the absence of IL-21 few if any cells with the phenotype of plasma cells were noted. This was most notable in cultures of cord blood B cells. Therefore, it is unlikely that IL-21 functions as a growth factor for plasmablasts induced by anti-IgM or/and anti-CD40 signaling, but rather plays an essential role in the decision to differentiate into nondividing, high-rate Ig-secreting cells. The capacity of IL-21 costimulation, but not anti-CD40 or anti-IgM alone, to induce BLIMP-1 mRNA is consistent with this conclusion.

The effects of other cytokines that bind γc-containing cytokine receptors on the actions of IL-21 were noteworthy. IL-2 has previously been shown to drive plasma cell differentiation when B cells are activated with SAC (30), as well as costimulate plasma cell differentiation in the presence of anti-CD40 and IL-10 (13, 25) or IL-6 (14, 17). IL-4, in contrast, has been shown to both promote and inhibit B cell responsiveness to various stimuli (13, 23, 45–

**FIGURE 8.** Ig production from IL-21-induced plasma cells is resistant to HU. Purified PB B cells were isolated and (A) cultured in the presence of IL-21 with or without anti-CD40 and anti-IgM and with or without HU (1 × 10−2 M) as indicated. After 3 days of culture, proliferation was determined by incubating the cells for 16 h with [3H]thymidine. B, Purified PB B cells were isolated and cultured with or without IL-21 in the presence of anti-CD40 and anti-IgM, and HU was added (or not) on the days indicated. The cultures were analyzed for IgD and CD38 expression by CD19+ B cells after 8 or 11 days in culture as indicated. C, Supernatants from IL-21, anti-IgM, and anti-CD40-stimulated cultures in B were analyzed for IgG content. HU was added where indicated. Data are representative of results from one of three experiments with similar results.
Most notable is the ability of IL-4 to inhibit SAC and IL-2-driven plasma cell differentiation (45, 47). We found that IL-2 enhanced the effect of IL-21 on plasma cell differentiation induced by the combination of anti-IgM and anti-CD40, whereas IL-4 inhibited it. These results suggest an antagonistic effect of IL-4 on the actions of IL-21 in B cells stimulated by anti-IgM or the combination of anti-IgM and anti-CD40. It is notable that antagonism between IL-4 and IL-21 was not consistently observed when cells were stimulated with anti-CD40 alone, indicating that the nature of the stimulus influences the ability of B cells to respond to the combination of IL-4 and IL-21. Moreover, the origin of the B cells may play a role because IL-4 has been shown to enhance Ig production by IL-21 and anti-CD40-activated CD27 human splenic memory B cells (26). These differences may reflect variability in the cytokine responsiveness of B cells from various compartments because IL-4 did not consistently inhibit or enhance anti-CD40 and IL-21-induced plasma cell differentiation by peripheral B cells. These findings make it unlikely that receptor competition can explain these results, owing to the fact that both IL-21 and IL-4 use the γc for signaling (49). In addition, IL-2, which also signals...
through a receptor-containing γc, reversed the IL-4 mediated-inhibition of IgM-producing plasma cell generation (data not shown), making the idea of receptor competition more remote. Furthermore, IL-2 synergized with IL-21 in fostering growth of anti-IgM and anti-CD40-stimulated B cells.

Taken together, these results suggest that the stimulus-dependent inhibition of IL-21 responses by IL-4 relates to the downstream signals generated by engagement of the IL-4R. In this regard, IL-4 is known to costimulate IgE synthesis by human B cells (48), and also induce the expression of CD23 on mature B cells (50). However, IL-4 also has been reported to repress human B cell responses (45, 47), and inhibit BLIMP-1 induction as well as plasma cell differentiation in mice (51). It is notable that in our studies, with the appropriate costimulation, IL-4 repressed BLIMP-1 expression, and inhibited plasma cell differentiation, whereas IL-21 induced both BLIMP-1 and plasma cell differentiation. In the appropriate signaling context, as discussed above, the actions of the two cytokines appear to be mutually inhibitory of one another. In this regard, IL-21 has been reported to inhibit proliferation of both murine and human B cells stimulated with anti-IgM and IL-4 (2, 7, 10) and to inhibit IL-4-induced IgE transcription (52). In this study, IL-4 repressed IL-21-driven plasma cell differentiation following either SAC or anti-IgM and anti-CD40 stimulation. It is of considerable interest that IL-4R ligation induces primarily STAT6, which is essential for IL-4 signaling, including IL-4-induced up-regulation of AID in both mice and humans (39, 40), whereas IL-21R engagement activates primarily STAT1 and -3 and more weakly STAT5 (1, 53, 54). Although the level of antagonism is unknown, the data suggest that IL-21 and IL-4 inhibit one another’s actions after receptor engagement. Biologically, T cell-dependent B cell responses may be regulated by T cells that either produce IL-21, IL-4, or other cytokines at
different times during an immune response, or different subsets of T cells that express different cytokine profiles. In support of this, IL-21 has been described to be produced by a restricted subset of follicular Th cells that express CXCR5 in humans (6).

Our final question was whether IL-21 had the ability to induce terminally differentiated nondividing plasma cells or merely dividing Ig-secreting plasmablasts. Ig-secreting cells have been shown to be quite heterogeneous, and different stages of plasma cell development have been described that can be identified phenotypically (55, 56). The majority of plasma cells in the germinal center are short lived, produce Ab rapidly and briefly, and then die in secondary lymphoid tissue. In contrast, a subpopulation matures into terminally differentiated nondividing plasma cells that exit lymphoid tissue and migrate to the bone marrow where they reside as long-lived plasma cells that produce Ab for long periods of time (55). These stages of plasma cells can be identified both functionally and phenotypically (56). It is notable that by phenotypic analysis, the IL-21-induced plasma cells could not clearly be identified as typical Ig-secreting plasma cells found in any one tissue compartment. Thus, they have some characteristic features of all plasma cells, including decreased expression of CD19, CD20, CD21, CD22, and CD23, as well as increased expression of CD27, CD38, and CD63. However, they have some features of tonsillar plasma cells, such as bright expression of CD9 and CD31 and decreased expression of CD49d, as well as some features of blood plasma cells, including bright expression of CD44 and CD62L. In addition, subpopulations of IL-21-induced plasma cells express features of bone marrow plasma cells, including diminished expression of HLA-DR and increased expression of CXCR4. The explanation for this mixed plasma cell phenotype is uncertain. It is not clear whether other features of the local microenvironment might contribute to the phenotype of resident plasma cells. Indeed, cytokines, such as B cell activation factor of the TNF family (BAFF) (57) or potentially IL-21 itself could affect the phenotype of plasma cells. Importantly, however, the plasma cells generated in response to IL-21 were postswitched nondividing, Ig-secreting cells as evidenced by their ability to continue to secrete Ig even when proliferation was inhibited by HU. These data, therefore, indicate that IL-21 costimulation drives naïve and memory B cells into terminally differentiated nondividing Ig-secreting plasma cells.

In conclusion, our studies demonstrate that IL-21 has pleiotropic effects on human B cells and strongly suggest that IL-21 is the major T cell-derived cytokine that drives plasma cell differentiation upon CD40 ligation. The ability of IL-21 to induce human plasma cell differentiation has implications not only in the treatment of immunocompromised individuals but also in autoimmune diseases where IL-21 blockade may have efficacy.

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Disclosures
The authors have no financial conflict of interest.

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