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Identification of Cellular Intermediates and Molecular Pathways Induced by IL-21 in Human B Cells

Danijela Konforte and Christopher J. Paige

The complex process of B cell development is controlled by multiple factors from the surrounding microenvironment including cytokines. IL-21 is a recently identified type I cytokine, mainly produced by activated CD4+ T cells. It has been shown to promote differentiation of human primary B cells into Ig-secreting plasma cells. The objective of our study was to describe cellular intermediates that exist during IL-21-induced transition from an activated B cell to an Ig-secreting cell and to identify molecular mechanisms involved in this process. Novel Epstein-Barr Virus-positive human B cell lines with phenotypes characteristic of Ag-activated IgG+ B cell blasts were used as a model system to study IL-21 effects in vitro. We show that IL-21 increased both proliferation and survival of B cell lines during the first 3 days of in vitro culture. This process was associated with CD38highCD19highCD20int cell surface phenotype. Continued culture with IL-21 resulted in accumulation of cells in G0/G1 stage of the cell cycle and increased apoptosis. This coincided with differentiation into small, CD38highCD20low/− cells that expressed lower levels of c-Myc protein, and secreted greater amounts of Ig than the control cells. Partial inhibition of IL-21-induced JAK/STAT signaling by the low-dose pharmacological agent, JAK inhibitor I, did not prevent the initial increase in proliferation. However, decrease in c-Myc protein expression and subsequent differentiation to late plasmablasts/early plasma cells were strongly inhibited. Our study is the first to show the link between IL-21-induced JAK/STAT signaling, c-Myc regulation, and differentiation of human B cells.


A network of cytokines produced by the lymphoid microenvironment plays a critical role in regulating functions of both the innate and the adaptive arms of the immune responses. Cytokines direct development and maturation of multiple immune cell lineages, control their functions, and terminate immune responses when the pathogen is cleared. The importance of the intact cytokine-induced signaling is underlined in multiple immunodeficiency diseases. For example, X-linked SCID results from mutations in the common cytokine receptor γ-chain (γc)3 that is shared by the receptors of IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (1, 2).

IL-21 is the most recently discovered member of the family of γc-dependent cytokines (3, 4). It is preferentially secreted by activated CD4+ Th cells (5, 6). Multiple reports point to the pleiotropic actions of IL-21 in the immune system. IL-21 was shown to enhance the proliferation and cytotoxicity of CD8+ T cells and to drive the differentiation and activation of NK cells (7–10). Such findings prompted multiple studies into the potential applications of IL-21 in immunotherapy. It has been shown that IL-21 has potent antitumor activity in several murine model systems including melanoma, adenocarcinoma, lymphoma, and fibrosarcoma where it enhances killing by tumor-specific T and NK cells (8, 11–13).

IL-21 plays an important role in regulating the proliferation, differentiation, and survival of murine B cells both in vivo and in vitro. For example, IL-21 increased growth and differentiation of murine B cells that received both BCR- and T cell help-mediated signals (3, 14, 15). By contrast, IL-21 induced growth arrest and apoptosis of LPS-stimulated murine B cells or of B cells that received a BCR-mediated signal in the absence of T cell help (14). Less is known about the effects of IL-21 on human B cells. A recent publication showed that IL-21 induced differentiation of CD40- and BCR-activated human naive and postswitch memory B cells and cord blood B cells into Ab-producing plasma cells (PCs) (16). In a malignancy setting, IL-21 enhanced growth of multiple myeloma cells and initiated apoptosis in chronic lymphocytic leukemia B cells (17, 18). Collectively, current data suggest that the effects of costimulation by IL-21 on murine and human B cells vary, so that the final outcomes of its signaling seem to depend on the activation state or on the developmental stage of the target cells. For these reasons, further insights into the identity and functions of IL-21-mediated signals in the biology of human B cells are required.

Post-germinal center (GC) B cells differentiate into high affinity Ig-secreting PCs or into memory B cells that are essential for protective immunity (19, 20). Our understanding of this process is largely derived from analysis of cells in one developmental stage or the other. It remains to be determined how transitions occur between the stages. The use of in vitro assays has permitted the study of B cell development in a controlled environment, allowing investigators to delineate discrete developmental stages and to identify molecular mechanisms involved in transitions between these stages. In this study, we developed an in vitro assay to track cellular intermediates that constitute IL-21-induced transition from...
an activated B cell to an Ig-secreting cell. We established novel EBV+ human B cell lines (BCLs) with characteristics of activated IgG+ B cells as determined by their transcription factor status and cell surface protein expression. These cells were used as a model system to study the effects of IL-21 in vitro. Our results demonstrate that exposure to IL-21 enhanced both proliferation and survival of BCLs during the first 3 days of in vitro culture. Longer exposure to IL-21 resulted in maturation into late plasmablasts/early plasma cells. These CD38hiCD23low/−/HLA-DR−/CD19hiCD20low cells had decreased expression of c-Myc, diminished growth and survival properties, and secreted higher levels of Ig than their precursors from day 3.

It has been reported that IL-21 induced activation of JAK/STAT signaling pathway in different cellular targets. However, the relative contribution of this pathway to IL-21-mediated developmental outcomes is not known. In this study, we show that partial inhibition of IL-21-induced JAK/STAT signaling did not prevent the initial increase in proliferation of B cells. By contrast, the intact JAK/STAT signaling was required for c-Myc repression and subsequent differentiation into late plasmablasts/early plasma cells.

Materials and Methods

**Cell lines and culture conditions**

EBV+ BCLs (BCL1 to BCL5) were established from EBV+ B cell populations that initially grew in coculture with syngenic fibroblasts isolated from five arthritis patients. Clonal BCLs were established from cells seeded at one cell per well. EBV+ diffuse large B cell lymphoma (DLBL) lines (OCI-Ly2, 4, 7, 10, 19) and multiple myeloma (MM) lines (My2, KMS11, KMS289E) were provided by the laboratories of H. Messner (OCILy2, 4, 7, 10, 19) and multiple myeloma (MM) lines (My2, KMS11, KMS289E) were provided by the laboratories of H. Messner (OCILy2, 4, 7, 10, 19) and multiple myeloma (MM) lines (My2, KMS11, KMS289E) were provided by the laboratories of H. Messner (OCILy2, 4, 7, 10, 19) and multiple myeloma (MM) lines (My2, KMS11, KMS289E) were provided by the laboratories of H. Messner (OCILy2, 4, 7, 10, 19) and multiple myeloma (MM) lines (My2, KMS11, KMS289E) were provided by the laboratories of H. Messner (OCILy2, 4, 7, 10, 19) and multiple myeloma (MM) lines (My2, KMS11, KMS289E) were provided by the laboratories of H. Messner (OCILy2, 4, 7, 10, 19) and multiple myeloma (MM) lines (My2, KMS11, KMS289E) were provided by the laboratories of H. Messner (OCILy2, 4, 7, 10, 19) and multiple myeloma (MM) lines (My2, KMS11, KMS289E) were provided by the laboratories of H. Messner (OCILy2, 4, 7, 10, 19) and multiple myeloma (MM) lines (My2, KMS11, KMS289E) were provided by the laboratories of H. Messner (OCILy2, 4, 7, 10, 19) and multiple myeloma (MM) lines (My2, KMS11, KMS289E) were provided by the laboratories of H. Messner (OCILy2, 4, 7, 10, 19) and multiple myeloma (MM) lines (My2, KMS11, KMS289E) were provided by the laboratories of H. Messner (OCILy2, 4, 7, 10, 19) and multiple myeloma (MM) lines (My2, KMS11, KMS289E) were provided by the laboratories of H. Messner (OCILy2, 4, 7, 10, 19) and multiple myeloma (MM) lines (My2, KMS11, KMS289E) were provided by the laboratories of H. Messner (OC(309,506),(692,877)
ELISA

ELISA plates (EIA/RIA plates; Corning Costar) were coated with 5 μg/ml affinity purified F(ab')2 rabbit anti-human IgA plus IgG plus IgM (H+L; Jackson ImmunoResearch Laboratories) in coating buffer (0.05 M Tris, 0.15 M NaCl (pH 9.5)) overnight at 4°C. Plates were washed and blocked for 1 h with 3% FCS in PBS and incubated with culture supernatants or human IgG and IgM (Sigma-Aldrich) isotype standards for 2 h at room temperature. After washing with water (eight times), plates were incubated for 2 h at room temperature with HRP-conjugated goat anti-human IgG (γ-chain specific) or HRP-conjugated goat anti-human IgM (μ-chain specific) (Sigma-Aldrich). Plates were washed and incubated with the TMB Microwell Peroxidase Substrate System (KPL). The reaction was stopped with 0.1 N HCL, and absorbance at 450 nm was read in a SpectraMax plate reader (Molecular Devices). To demonstrate isotype specificity, purified IgM isotype (Sigma-Aldrich) were run in the IgG ELISAs and produced no signal (data not shown). The limit of sensitivity of the IgG ELISA was 2 ng/ml.

Cell staining

To visualize cytoplasmic and nuclear morphology, cytospins (8 × 10⁴ cells) were fixed in methanol and stained with LeukoStat Solution I (eosin Y 0.1% w/v) and II (methylene blue 0.047% w/v and azure A 0.044% w/v; Fisher Diagnostics) according to manufacturer’s guidelines. Cell images were visualized with a Nikon OPTIPHOT upright brightfield microscope using the objective lens by Olympus Dplan ×20/0.4. The microscope was connected to a CoolSNAP-ProCF color camera (Roper Scientific Photometrics) and images were acquired with Media Cybernetics Image Pro PLUS software.

Results

Establishment and characterization of EBV⁺ BCLs

We cloned BCLs from populations that were established from in vitro cultures of synovial cells obtained from five arthritis patients. All cell lines were positive for EBV. RT-PCR and immunostaining results indicated that these cells expressed EBV nuclear Ags, ebna2 and ebna3c, and latent membrane proteins, lmp1 and lmp2a. They also expressed zta and rta, immediate early genes of the lytic cycle (data not shown). This profile of viral gene expression is typical of type III viral latency (21).

FIGURE 1. Gene expression profile and cell surface phenotype of EBV⁺ BCLs. A, RT-PCR analysis of bcl6, pax5, blimp1, and aid in DLBL (-1, -2), EBV⁺ BCLs (-3, -4), and MM (-5, -6) lines. Two representative lines from each group are shown. B, Real-time RT-PCR analysis of xbp1 expression normalized to gapdh. Both spliced and unspliced forms of xbp1 gene were detected by the same set of primers. Error bars are SD of triplicate wells; p = 0.040 between DLBL and EBV⁺ BCLs, and p = 0.053 between EBV⁺ BCLs and MM cell lines. Values of p are calculated from two-tailed, unpaired t test where n = 5 for each group of lines. C, FACS analysis of indicated surface markers. Histograms are representative of five lines tested in each group. EBV⁺ BL stands for EBV-positive Burkitt’s lymphoma line.

FIGURE 2. IL-21R (IL-21R) surface expression on BCLs decreases in response to IL-21. A, Surface expression of IL-21R on BCLs was examined by FACS. Open histograms represent binding of mouse IgG1,κ monoclonal anti-human IL-21R-PE while closed histograms represent binding of irrelevant mouse IgG1,κ mAb. B, BCL1 was cultured with IL-21 (50 ng/ml) for 7 days. On indicated days, control and IL-21-cultured cells were analyzed for changes in the surface expression of IL-21R by FACS. FACS was performed as described in A. BCL1 is representative other four lines.

FIGURE 3. IL-21 increases tyrosine phosphorylation of JAK1, JAK3, STAT1, and STAT3 in BCLs. Cell lysates were collected at indicated times after addition of IL-21 to the culture. Tyrosine-phosphorylated forms of JAK1 (pY1022/pY1023), JAK3 (pY980), STAT1 α and β (pY701), STAT3 (pY705), and ERK1/2 (pThr202/pY204) proteins were detected by Western blotting. Total ERK demonstrates equal protein loading.
We further characterized these cell lines by determining patterns of gene expression that mark the progression of cells along the B cell pathway. EBV-negative BCLs that belong to well-defined stages of mature B cell differentiation, including large DLBL lines with GC-like phenotype and MM lines with PC-like phenotype were included in this analysis. RT-PCR results suggested that BCLs were either a homogeneous group of cells that exhibited features of both Ag-activated B cells and more differentiated plasma cell precursors, or were developmentally heterogeneous, with some cells committed to PC differentiation (i.e., bcl6/H11002, blimp1/H11001, xbp1int) while others remained in a less differentiated state (i.e., aid/H11001, pax5/H11001) (Fig. 1, A and B). To distinguish between these possibilities, we used FACS analysis and found that BCLs exhibited a homogeneous pattern of expression of several cell surface proteins including the presence of surface Ig H chain (sIgH), CD20, CD19, but the absence of a GC marker, CD10, and a PC marker, CD138 (Fig. 1C). In addition, BCLs expressed memory cell marker CD27. These cells were negative for chemokine receptors CXCR5 and CXCR4 that are usually expressed by GC and Ig-secreting B cells, respectively (data not shown). All cell lines were IgG1 positive indicating that they have undergone isotype switching (D. Konforte and C. J. Paige, unpublished data). In-frame Ig VH chain (VH) gene rearrangements that belonged to functional VH gene families were found in all tested lines by amplifying and sequencing their VH-DH-JH-C\(\gamma\)1 cDNA regions (data not shown).

IL-21R α-chain (IL-21R) expression on the surface of BCLs decreases in response to IL-21

Before examining the effects of IL-21 on BCLs, we tested for the expression of IL-21R in these cells. Real-time quantitative PCR data indicated that IL-21R message was present in all cell lines (data not shown). Low and homogeneous cell surface expression of IL-21R was detected by flow cytometry on all cell lines. Jurkat cell line was used as a negative control for IL-21R expression (Fig. 2A). Addition of exogenous IL-21 to the cell culture caused a gradual decrease in the surface expression of IL-21R. By day 4, IL-21R was undetectable on the surface of BCL1 (Fig. 2B).
IL-21 induces tyrosine phosphorylation of JAK1, JAK3, STAT1, and STAT3 in BCLs

It has been reported that binding of IL-21 to its receptor (IL-21Rα and γc) initiates signaling by activation of the JAK/STAT pathway (1, 22). Western blot analysis of tyrosine phosphorylation status of JAK and STAT molecules was undertaken to further address the functionality of IL-21R on BCLs. With the exception of JAK3, we did not detect significant levels of constitutive tyrosine phosphorylation of JAK1 or STATs in any of BCLs. Increase in tyrosine phosphorylation of JAK1, JAK3, STAT1, and STAT3 molecules was detected after addition of IL-21 to the culture (Fig. 3). Unlike STAT1, tyrosine phosphorylation of STAT3 remained elevated even 60 min after addition of IL-21 to the cultures. Sixty minutes of exposure to IL-21 did not change the phosphorylation status of the MAPKs ERK1 and 2 (p44/p42 MAPK) that were constitutively active in BCLs (Fig. 3).

Kinetic analysis of the effects of IL-21 on growth and viability of BCLs

Inclusion of IL-21 during 3-day in vitro culture resulted in increased proliferation of BCLs in a dose-dependent fashion, as determined by [3H]thymidine incorporation assay (Fig. 4A). To determine whether these changes translated into increased total cell numbers, BCLs were cultured with 50 ng/ml human recombinant IL-21 on day 0. Every 3 to 4 days, control and IL-21-treated wells were harvested, counted by trypan blue exclusion method, and placed back in culture at the cell concentration equal to that on day 0. Cells were cultured in fresh medium with or without IL-21. Fold cell expansion relative to day 0 was calculated for the indicated time periods (A, control cells; B, IL-21-stimulated cells). B, Fold percentage (%) dead cells stands for percentage of trypan blue positive cells in wells with IL-21 divided by percentage trypan blue positive cells in the control wells.

Table I. Summary of percentages of BCLs in different stages of cell cycle on day 3 and day 6 of culture with IL-21a

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<tr>
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<th>Day 3</th>
<th>Day 6</th>
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<tr>
<td></td>
<td>Control (G0/G1)</td>
<td>Control (G0/G1)</td>
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<tr>
<td>BCL1</td>
<td>58.51</td>
<td>63.94</td>
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<tr>
<td></td>
<td>38.42</td>
<td>33.31</td>
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<td>3.07</td>
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<td></td>
<td>24.74</td>
<td>19.64</td>
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<td>5.38</td>
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<td>BCL3</td>
<td>64.04</td>
<td>67.86</td>
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<td></td>
<td>31.10</td>
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<td></td>
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<td>4.76</td>
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<tr>
<td>BCL4</td>
<td>70.44</td>
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<td></td>
<td>26.14</td>
<td>22.77</td>
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<td></td>
<td>3.43</td>
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a PI-labeled DNA content was measured by FACS and analyzed by ModFit LT software. Apoptotic cells were not included in the analysis. The results are representative of two independent experiments.

FIGURE 5. Effects of IL-21 on growth and survival of BCLs during prolonged in vitro culture. A, BCL1, 3, and 4 were stimulated with 50 ng/ml human recombinant IL-21 on day 0. Every 3 to 4 days, control and IL-21-treated cells were harvested, counted by trypan blue exclusion method, and placed back in culture at the cell concentration equal to that on day 0. Cells were cultured in fresh medium with or without IL-21. Fold cell expansion relative to day 0 was calculated for the indicated time periods (A, control cells; B, IL-21-stimulated cells). B, Fold percentage (%) dead cells stands for percentage of trypan blue positive cells in wells with IL-21 divided by percentage trypan blue positive cells in the control wells.

Cells in G0/G1 phase of the cycle. For example, between days 3 and 7 of culture with IL-21, the proportion of BCL1 cells in G0/G1 phase increased from 51 to 70% (Table I). This coincided with a reduction in cell size. Forward scatter profiles indicated that the majority of IL-21-cultured cells were smaller in comparison to the control cells on days 4 and 6 of culture (data not shown).

c-Myc is a transcription factor expressed in almost all proliferating cells, and its repression is required for cell cycle exit in many cell types, including B cells (25, 26). Consistent with these results, we show that the expression of c-Myc protein was strongly decreased by day 6 of culture with IL-21 (see Fig. 4D). At the same time, the expression of p27Kip1 increased (Fig. 4D). p27Kip1 is a direct target of repression by c-Myc. It acts as a cyclin-dependent kinase inhibitor by interfering with the activity of cyclin E-CDK2 complexes and transition from the G1 to S phase of the cell cycle (27).

Annexin V/PI staining of BCL2 shows that addition of IL-21 to the culture decreases the proportion of apoptotic cells on days 2 and 4 (Fig. 4E). The total number of BCL2 cells counted in the control wells and IL-21-treated wells on day 4 was 7 × 10^5 and 14 × 10^5, respectively (data not shown). Therefore, 43% of annexin V+ and annexin V+PI+ control cells translates into 3.0 × 10^5 cells, while 18% of annexin V+ and Annexin V+PI+ IL-21-treated cells translates into 2.5 × 10^5 cells which equals to a 1.2-fold decrease in the number of dead cells. This suggests that the increase in the cell numbers on day 3 (Fig. 4B) or day 4 is mostly due to IL-21-mediated increase in proliferation (Fig. 4C and Table I).
Reduced cell growth and viability of BCLs was observed with the inclusion of IL-21 for longer periods of in vitro culture (up to day 22) (Fig. 5).

In all of the above experiments, BCLs were washed, counted, and replated at $2 \times 10^5$ cells/4 ml of fresh medium with IL-21 (50 ng/ml) every 3 days throughout the culture. This excluded the possibility that cell overcrowding caused it was previously shown that Bcl6 represses proliferation (28). Blimp1-regulated TFs involved in B cell differentiation such as unspliced-xbp1 and irf4 also increased in response to IL-21. The spliced form of xbp1 was not detected (data not shown). Gene expression of TFs regulating B cell identity, such as blimp1, aidlowbcl6, and HLA-DR) (Fig. 6A), CD23 expression was almost completely extinguished on day 6. Decrease in CD19, HLA-DR, and slgM was not detected until day 4 of culture. Interestingly, surface expression of a differentiation marker, CD38, decreased between days 0 and 2, and then gradually increased by day 6 of culture.

In addition, BCLs acquired morphological characteristics of plasmacytoid cells with increased cytoplasmic content, eccentric nuclei, and light perinuclear zones consistent with expanded Golgi membranes (Fig. 6B).

We also analyzed changes in gene expression patterns of TFs that are critical during the process of late B cell differentiation. RT-PCR results show that exposure to IL-21 for 6 days resulted in an increased expression of blimp1 mRNA in BCLs (Fig. 6C). Blimp1 is a master TF that triggers plasma cell differentiation through coordinated repression of genes associated with the B cell phenotype and cellular proliferation (28). Blimp1-regulated TFs involved in B cell differentiation such as unspliced-xbp1 and irf4 also increased in response to IL-21. The spliced form of xbp1 was not detected (data not shown). Gene expression of TFs regulating B cell identity, such as pac5 and aid, were decreased by IL-21 (Fig. 6C). It was unexpected to find that bcl6 mRNA expression increased in response to IL-21 (Fig. 6C) because it was previously shown that Bcl6 represses blimp1 (29).

Ig (nanograms per milliliter) in the supernatants from the control and IL-21-containing cultures was measured by ELISA. BCLs constitutively secreted low levels of Ig. Although there was no difference in the amount of Ig in the supernatants from the control and IL-21-containing cultures during the first 3 days of culture, a significant increase in the amount of secreted Ig was detected during the subsequent culture with IL-21 (Fig. 7).

Collectively, our results show that after 6 days of culture with IL-21, a proportion of BCLs accumulated in G0/G1 stage of the cell cycle, acquired CD38highCD23lowHLA-DRlowCD19unblimp1highirf4highpax5unlowaidunbcl6un phenotype, and secreted higher levels of Ig, all of which are consistent with the progression to late plasmablast/early plasma cell stage of development. BCLs maintained this phenotype (data not shown) and continued to divide during the long-term culture with IL-21, albeit at a slower rate, suggesting that additional factors, apart from IL-21, are required for their transition into resting PCs or memory cells.

**FIGURE 6.** IL-21 induces differentiation of BCLs to late plasmablasts/early PCs. **A,** Expression of cell surface markers on control cells (filled histograms) vs IL-21-treated cells (open histograms) were compared by FACS on days 2, 4, and 6 of culture with IL-21. For each plot, histograms represent the fluorescence of cells incubated with specific or control mAbs. BCL3 is representative of four BCLs tested. B, Day 10 cytospins of control and IL-21-treated BCL3 cells were stained with eosin and methylene blue. Cell images were captured at $\times 20$ magnification. C, RT-PCR analysis of a panel of genes in control and IL-21-treated BCL1 and BCL3 lines on day 6 of culture. Numbers represent the fold change in gene expression after normalization to actin.

**FIGURE 7.** Kinetic analysis of changes in the level of Ig secreted by BCLs in response to IL-21. A total of $2 \times 10^5$ of BCLs were cultured in 4 ml of medium supplemented with IL-21. Every 3 days until day 22, culture supernatants were collected, and the cells were counted, washed, and placed back in culture at $2 \times 10^5$ cells/well in fresh medium containing IL-21. Ig (nanograms per milliliter) in the supernatant was detected by ELISA and normalized to the number of cells recovered at the time of supernatant collection to obtain the average ng of secreted Ig per cell. Fold change for IL-21-treated vs control cells was calculated for indicated days.

**IL-21 induces maturation of BCLs into Ig-secreting late plasmablasts/early plasma cells**

Next, we analyzed the expression patterns of cell surface molecules that have been reported to change during the B cell differentiation process. FACS analysis of the cell surface phenotype from days 2, 4, and 6 of culture with IL-21 revealed a time-dependent change in the expression of mature B cell markers (CD19 and Ig H chain, IgM) and of activation-associated markers (CD23 and HLA-DR) (Fig. 6A), CD23 expression was almost completely extinguished on day 6. Decrease in CD19, HLA-DR, and slgM was not detected until day 4 of culture. Interestingly, surface expression of a differentiation marker, CD38, decreased between days 0 and 2, and then gradually increased by day 6 of culture.

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A pyridone-containing tricyclic, JAKi I, opposes the decrease in proliferation and survival of BCLs cultured with IL-21.

Consistent with the previous results from different cellular backgrounds, we showed that IL-21 induced tyrosine phosphorylation of JAK1, JAK3, STAT1, and STAT3 in BCLs (Fig. 3). However, the relative contribution of JAK/STAT signaling to IL-21-mediated B cell development has not been studied. We tested the effect of a pharmacological agent, JAKi I, on IL-21-mediated growth and survival of BCLs. In vitro kinase assays that were used to test the specificity of JAKi I showed that it inhibited kinase activity of JAKs at concentrations lower than 130 nM, but lost its specificity for JAKs, and inhibited multiple kinases at higher concentrations (30). BCLs...
were incubated with multiple doses of JAKi I (10 –1000 nM) for 45 min before addition of IL-21 to the cultures. Cell lysates were collected at indicated times after addition of IL-21 to the culture. Tyrosine-phosphorylated STATs were detected by Western blotting as described in Fig. 3. Total STAT1 and STAT3 demonstrate equal protein loading. C. BCL5 cells were cultured as in A and B. c-Myc and p27Kip1 expression was detected in nuclear lysates by Western blotting. Actin demonstrates equal protein loading. Intensity of bands was normalized to total STAT3 in A, total STAT1 and STAT3 in B, and actin in C. Numbers represent the fold change in STAT phosphorylation after normalization to total STAT3 in A, total STAT1 and STAT3 in B, and the fold change in c-Myc and p27Kip1 expression after normalization to actin in C.

Low-dose JAKi I strongly inhibits IL-21-induced tyrosine phosphorylation of STAT1 and STAT3, and prevents decrease in c-Myc and p27Kip1 protein expression

To confirm that JAKI I specifically inhibited JAK kinase activity, we analyzed changes in tyrosine phosphorylation of STAT molecules by Western blot. Cell lysates were collected 5, 35, and 65 min after addition of IL-21 to the cultures that were preincubated either with JAKI I (200 nM) or with DMSO (control) for 45 min. IL-21-induced tyrosine phosphorylation of STAT1 and STAT3 was significantly inhibited by JAKI I (Fig. 9A). A similar pattern of inhibition was present on day 3 and on day 6 of culture with IL-21. Secreted Ig per cell was detected by ELISA as described in Fig. 7.
IL-21 (Fig. 9B). Notably, at all time points, the inhibition of tyrosine phosphorylation of STAT1 was stronger than that of STAT3.

In addition, JAKi I interfered with the inhibition of c-Myc protein expression on days 3 and 6 with culture with IL-21 (Fig. 9C). Consequently, increase in p27Kip1 protein was also partially inhibited on day 6 (Fig. 9C). These results suggest that the IL-21-induced JAK/STAT pathway could play an important role in cell cycle progression by regulating c-Myc and p27Kip1 expression in B cells.

IL-21-induced differentiation of BCLs into late plasmablasts/early plasma cells is strongly diminished in the presence of JAKi I

We hypothesized that the insufficient decrease in c-Myc expression in the presence of JAKi I ensured continued proliferation of BCLs that could interfere with IL-21-mediated differentiation into late plasmablasts/early plasma cells. FACs analysis of the surface phenotype of BCLs indicated that the changes consistent with B cell differentiation were strongly inhibited by JAKi I. Two-color FACs showed that maturation into CD38highCD23low/− population that represented 74% of IL-21-treated cultures on day 6 was reduced to 16% in the presence of JAKi I (Fig. 10A). Decrease in the expression of CD20 was partially prevented, while decrease in the expression of CD19−DR, CD19, and slgK was completely prevented by the inhibitor (data not shown). In addition, ELISA results indicated that treatment with JAKi I prevented IL-21-mediated increase in secreted Ig (Fig. 10B).

IL-21 induces tyrosine phosphorylation of JAK1, JAK3, STAT1, and STAT3 in primary human B cells activated with anti-CD40 and anti-IgM

To confirm that IL-21-induced signaling pathways in BCLs also apply to primary human B cells, we isolated CD19+ B cells from the PB of four healthy donors and activated them with anti-IgM and anti-CD40 in the presence or absence of IL-21. Anti-IgM and anti-CD40 were used to activate B cells in vitro because this combination most closely resembles B cell activation via Ag and T cell interaction, respectively. Western blot results showed that, similarly to BCLs, IL-21 strongly induced tyrosine phosphorylation of JAK1, STAT1, and STAT3 molecules in activated primary B cells. This was detected on day 0 at 20 min, and 40 min after addition of IL-21 to the culture (Fig. 11A). Tyrosine phosphorylation of JAK3 was not observed on day 0. It was, however, detected in B3 and B4 samples on day 1 when cells were activated with anti-CD40 and anti-IgM for 12 h before addition of IL-21 (Fig. 11B). The overall phosphorylation signals were stronger when IL-21 was added to the culture 12 h after activation of cells with anti-CD40 and anti-IgM. Based on the previous reports, it is reasonable to speculate that this is due to increased expression of IL-21R on the surface of activated B cells. Similarly to BCLs, activation of JAK and STAT molecules was observed even 3 days after addition of IL-21 to the culture. IL-21 had no effect on phosphorylation of ERK1/2 in B cells after 20 or 40 min of culture, which also agrees with the results obtained in BCLs (Fig. 11B). Difference in the intensity of bands between the samples at different time points is due to the difference in the total number of cells that were lysed and loaded in each lane.

Discussion

In vitro studies have contributed greatly to our understanding of the terminal stages of human B cell development, and of the role of cytokines in this process. It has been shown that Ig-secreting cells can be obtained in vitro by culturing human GC or memory B cells with bone marrow stromal cells and/or activated T cells, or the other T cell-derived stimuli such as CD40L, IL-2, and IL-10 (31–35). We developed an in vitro assay to characterize the critical events that constitute the transition from activated B cells to Ig-secreting late plasmablasts/early plasma cells in response to exogenous IL-21. Here, we show that this transition can be broken down into proliferation- and survival-inducing signals (days 0–3) followed by differentiation-inducing signals that require an intact JAK/STAT-signaling pathway.

Human EBV+ BCLs that express viral proteins characteristic of type III viral latency were used as a model system to study the effects of IL-21 in vitro. EBV+ BCLs were cloned from oligo-clonal B cell populations that expanded in cultures with autologous synovial tissue-derived fibroblasts. These cells likely arose by activation of latently infected memory B cells that were allowed to enter the GCs or the extrafollicular areas of the lymph nodes, tonsils, or spleen (35, 36). EBV+ BCLs constitutively express intermediate levels of a PC transcription factor, blimp1, without having completely extinguished the mature B cell expression profile (CD19highCD20highpax5+aid+).

FIGURE 11. IL-21 induces tyrosine phosphorylation of JAK1, JAK3, STAT1, and STAT3 in primary CD19+ PB B cells stimulated with anti-CD40 and anti-IgM. A. On day 0, purified CD19+ PB B cells were stimulated with anti-CD40 and anti-IgM in the absence (−) or presence (+) of IL-21, and cell lysates were collected at indicated times. B, CD19+ PB B cells from B3 and B4 samples were stimulated with anti-CD40 and anti-IgM 12 h before addition of IL-21. Cell lysates were collected at indicated times. Western blotting was performed as described in Fig. 3. Actin demonstrates equal protein loading between control and IL-21-treated cells for each sample.
Most EBV⁺ BCLs with type III viral latency are established by in vitro infection of PB B cells with the recombinant strain of EBV. It has been reported that these cells remain blocked at the lymphoblastoid stage and continue to proliferate indefinitely (37). By contrast, we show that the synovial tissue-derived EBV⁺ BCLs retained the capacity to alter their growth properties and differentiate in response to exogenous IL-21. Despite some variability in the timing and the magnitude of the responses to IL-21, all BCLs behaved in a similar fashion. After 3 days of culture with IL-21, these cells acquired CD38low/int/CD23low/HLA-DRhigh/CD19high/CD20low surface phenotype, displayed an increased rate of cell division, and increased survival compared with the control cells. At this time, they continued to secrete low levels of Ig similar to the controls. Collectively, these results suggest progression into the proliferating plasmablast stage of development. Even though Annexin V/PI staining on day 3 indicated reduction in the percentage of apoptotic cells in culture with IL-21, the difference in the absolute numbers of annexin V⁺ and Annexin V⁺/PI⁻ BCL2 cells in the absence or presence of IL-21 was small. This suggests that the IL-21-mediated increase in proliferation is the major mechanism responsible for the increase in cell recovery on day 3. Subsequent maturation of BCLs into late plasmablasts/early PCs was associated with the accumulation of cells in the G2/M phase of the cell cycle, decreased survival, and an increased amount of secreted Ig. Reduced proliferation on day 6 could be explained by the lowered responsiveness of BCLs to IL-21 caused by the loss of expression of the IL-21R on the cell surface. In support of this, the FACS analysis showed complete loss of the IL-21R on the surface of BCL1 on day 4, which preceded reduction in proliferation and differentiation on day 6.

Late plasmablasts/early PCs displayed c-Myclow/p27Kip1high CD38high/CD23low/HLA-DRhigh/CD19high/CD20low (blimp1high/irf4high pax5low/aidlow/bcl6⁻) phenotype. Reduction of CD23 surface expression on BCLs was one of the most striking phenotypic changes caused by IL-21. CD23, a low-affinity receptor for IgE (FcεRII), is a type II integral membrane glycoprotein with the extracellular sequence containing a C-type lectin domain (38). Our results are in agreement with the previous findings that CD23 surface expression is high on Ag-activated B cells, while it decreases with differentiation to plasma cells (39). IL-21-induced decrease of CD23 on BCLs may indicate the reduction of Ag presentation functions as activated B cells differentiate toward the PC fate. CD23 is known to be noncovalently associated with HLA-DR molecules on activated B cells. This interaction is thought to be involved in the process of recycling between the cell membrane and endosomal network that contributes to the efficiency of Ag presentation to T cells. In addition, the association of CD23 with HLA-DR at the surface of B cells may help to stabilize B cell-T cell interactions, contributing to T cell activation (38). Our results show that both HLA-DR and CD23 surface expression on BCLs decreased in response to IL-21. This data and the previous reports could suggest that the expression of HLA-DR and CD23 is coregulated during B cell development. Additionally, decrease in CD23 surface expression on BCLs could be a consequence of IL-21-mediated reduction of pax5 expression. Pax5 was found to be a key regulator of CD23 expression in B cells (40).

In contrast to a previous report by Ettinger et al. (16) who showed that IL-21 increased aid expression in human B cells, we detected a decrease in aid expression in BCLs. The most likely reason for this discrepancy is the timing of the experiments. Ettinger et al. (16) examined changes in aid expression on day 3 of culture with IL-21. This preceded differentiation of B cells to PCs on day 7. We, in contrast, examined aid expression on day 6, when BCLs already acquired characteristics of differentiated, late plasmablasts/early PCs. aid expression is known to decrease as B cells differentiate toward the PC fate. Second, the differences could reflect differences in the stage of development of B cells at the onset of in vitro culture. Ettinger et al. (16) used CD19⁺ primary B cells from the PB that contained mostly of IgD⁻ naïve B cells (between 67 and 78%). It is likely that an increase in aid expression is mostly due to naïve IgD⁺ cells that undergo isotype switching from IgM to IgG. They do not show changes in aid expression in response to IL-21 that is specific to CD27⁺ IgD⁺ IgG⁻ switched memory cells. This population would be more equivalent to B cells used in our experiment where EBV⁺ BCLs are isotype-switched IgG⁺ B cells resembling Ag-activated memory B cells. It is possible that IL-21 affects aid expression differently in activated naive vs activated switched memory B cells.

The apparent differentiation to late plasmablasts/early PCs could depend on the preferential expansion of a subset of partially differentiated plasmablasts. Homogenous expression of IL-21R on cells within each cell line diminished the possibility of the selection of a subset of cells that are particularly sensitive to the early IL-21-driven proliferation due to higher expression of IL-21R. Some BCLs showed heterogeneous expression of a differentiation marker CD38 (data not shown). We sorted CD38low/int and CD38high populations and compared their responsiveness to IL-21. Both populations responded similarly, by increased proliferation followed by differentiation into late plasmablasts/early PCs (data not shown).

Cytokines that regulate B cell development such as IL-10, IL-6, IL-4, IL-2, and IL-21 initiate signaling by activating the JAK family protein tyrosine kinases (JAK1–3, Tyk2) (41–43). Activated JAKs mediate cytokine-dependent activation of STATs (STAT1–6) by phosphorylating tyrosine residues in STATs. Activated STATs then dimerize and translocate into the nucleus where they activate and/or repress transcription of array of genes whose functions will determine cell fates during the subsequent development (43). Our study provides novel insights into the function of JAK/STAT signaling pathway in IL-21-induced B cell development. We show that the partial inhibition of tyrosine phosphorylation of STAT1 and STAT3 by low-dose JAKI (≤250 nm) was not sufficient to prevent IL-21-mediated increase in proliferation during the early culture. However, subsequent differentiation into late plasmablasts/early PCs was strongly reduced. Our results suggest that failure of IL-21 to repress c-Myc protein expression in the presence of JAKI could, at least in part, be responsible for the continued proliferation of BCLs and inhibition of their differentiation into late plasmablasts/early PCs on day 6. Our data support previous studies which indicated that the exit from cell cycle via c-Myc repression was a mechanistic requirement for the transition from the proliferating B cell to Ig-secreting plasma cell stage (19, 44, 45).

It has been reported that ectopic expression of Blimp1 is sufficient to inhibit endogenous c-Myc, and to drive differentiation of murine BCL1 cells to PC stage (46). Interestingly, our RT-PCR results revealed that the IL-21-mediated increase in blimp1 on day 3 and on day 6 of culture was not significantly inhibited by JAKI (data not shown). Continued expression of c-Myc despite elevated blimp1 suggests that factors other than Blimp1 regulate expression of c-Myc downstream of IL-21. Our in vitro assay will be an excellent tool to identify and study negative regulators of cell cycle progression that act downstream of IL-21-induced JAK/STAT signaling pathway.

Data from the JAKI study also suggest that multiple signaling pathways could play a role in early IL-21-induced proliferation of B cells, and could compensate for the lack of intact JAK/STAT signaling. Three major signaling pathways activated by γc cytokines are the PI3K/Akt, RAS-MAPK, and JAK/STAT pathways
IgM determined whether, similarly to other members of subsequent proliferation of B lineage cells (52). It remains to be phenylalanine prevents recruitment of p85 subunit of PI3K and IL-4R MAPKs are involved in the mitogenic responses to IL-21. PI3K, by BCLs. It remains to be determined whether pJNK and p38 against its involvement in the early enhancement of proliferation tect increase in the level of phosphorylation of ERK1/2 on threo-tribute to IL-21-mediated proliferation of B cells. We did not de-

apoptosis and exhibited increased survival and proliferation in Splenocytes from STAT1-deficient mice were less susceptible to been implicated in cell cycle arrest and apoptotic cell death (55). STAT1 and STAT3 have been shown to have opposing effects on depend on the balance between the activities of STAT1 vs STAT3. IL-21R. to link them with developmental outcomes in B cells. The ability to determine whether IL-21 induces B cell costimulation, growth arrest, or Bim-
determine whether IL-21 mediated proliferation of BCLs continue to dominate in the later cul-

disorders such as systemic lupus erythematosus and rheumatoid arthritis where the plasma cell responses are deregulated.

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Disclosures

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14. Jin, H., R. Carrio, A. Yu, and T. R. Malek. 2004. Distinct activation signals in primary B cells expressing STAT1 (57). In contrast to STAT1, STAT3 mostly functions as an antiapoptotic factor, especially in numerous malignancies, where it is constitutively activated/phosphorylated (53, 58, 59). It was demonstrated that STAT3 can function as an oncogene and is able to transform normal fibroblast cells and cause tumors in nude mice (54). Dominant negative or antisense STAT3 constructs induced apoptosis in several tumor cell lines (60). IL-21 was shown to act as a long-term growth and survival factor in MM lines where only STAT3 but not STAT1 was activated (17). Our results show that throughout the culture with IL-21 (days 0–6), tyrosine phosphorylation of STAT1 was more strongly inhibited by JAKI I than tyrosine phosphorylation of STAT3. We propose that in the absence of sufficient STAT1 signals, growth- and survival-promoting STAT3 signals that contribute to early IL-21-mediated proliferation of BCLs continue to dominate in the later culture, thereby preventing decrease in c-Myc expression necessary for the cell cycle exit, and consequent differentiation to late plasmablasts/early PCs.

Results obtained with cell lines need to be interpreted cautiously with respect to extrapolation to mechanisms in normal cells. Our study shows that in culture with IL-21, EBV + BCLs responded in a fashion similar to primary human B cells stimulated with anti-IgM and anti-CD40 (16). Furthermore, we showed that IL-21-induced phosphorylation of JAK1, JAK3, STAT1, and STAT3 detected in BCLs also applies to activated primary B cells from the PB. This result lends validity to the use of BCLs as an appropriate in vitro model for studying developmental outcomes that follow BCR- and T cell-mediated activation of human B cells. The in vitro system described in this report provides a valuable tool to thoroughly dissect molecular mechanisms of IL-21 signaling and to link them with developmental outcomes in B cells. The ability to manipulate IL-21 signals in B cells, either by disrupting or amplifying them, could have clinical benefit both in malignancies and in autoimmune disorders such as systemic lupus erythematosus and rheumatoid arthritis where the plasma cell responses are deregulated.

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