The Journal of Immunology

Analysis of the Role of IL-21 in Development of Murine B Cell Progenitors in the Bone Marrow

Nathalie Simard, Danijela Konforte, Anne H. Tran, Jessica Esufali, Warren J. Leonard and Christopher J. Paige

J Immunol 2011; 186:5244-5253; Prepublished online 23 March 2011;
doi: 10.4049/jimmunol.1004040
http://www.jimmunol.org/content/186/9/5244

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Analysis of the Role of IL-21 in Development of Murine B Cell Progenitors in the Bone Marrow

Nathalie Simard,*,†,[1] Danijela Konforte,*,[1] Anne H. Tran,* Jessica Esufali,* Warren J. Leonard,‡ and Christopher J. Paige*,†,§

IL-21 plays a key role in the late stage of B cell development, where it has been shown to induce growth and differentiation of mature B cells into Ig-secreting plasma cells. Because IL-21R has also been reported on bone marrow (BM) B cell progenitors, we investigated whether IL-21R influenced earlier stages of B cell development. IL-21R is functional as early as the pro-B cell stage, and the strength of receptor-mediated signaling increases as cells mature. The addition of IL-21 to B cell progenitors in cell culture resulted in the accelerated appearance of mature B cell markers and was associated with the induction of *Aid*, *Blimp1*, and germline transcripts. We also found that stimulation of both IL-21R and CD40 was sufficient to induce the maturation of early B cell progenitors into IgM- and IgG-secreting cells. Consistent with a role for IL-21 in promoting B cell differentiation, the number of $B220^+CD43^+IgM^-$ pro-B cells was increased, and the number of mature $IgM^{hi}IgD^{hi}$ cells was decreased in BM of IL-21R–deficient mice. We also report in this paper that IL-21 is expressed by BM $CD4^+$ T cells. These results provide evidence that IL-21R is functional in B cell progenitors and indicate that IL-21 regulates B cell development. ***The Journal of Immunology*****, 2011, 186: 5244–5253.**

The generation of mature, Ag-responsive B cells from pluripotent stem cells is directed by signals provided by the supportive microenvironment in the bone marrow (BM). Some of these signals are mediated by the action of soluble cytokines. At each developmental stage, B cells express a distinct profile of cytokine receptors on their cell surface to sense these signals. In mice, one critical phase of B cell development in the BM is regulated by the cytokine IL-7. It has been established that IL-7 promotes proliferation, survival, and development of pro-B cells toward the pre-B cell stage (1–4). Furthermore, our laboratory has previously shown that once the pre-BCR is expressed at the cell surface, integrated signals from both the IL-7R and the pre-BCR allow cells to proliferate in reduced concentrations of IL-7 (5).

IL-7 is a member of the common γ chain-dependent cytokine family, which includes IL-2, IL-4, IL-9, IL-15, and IL-21 (6). Like IL-7, IL-21 has also been shown to play a key role in B cell development. However, in contrast to IL-7, current data show that IL-21 exerts its effects at later stages when B cells differentiate into plasma cells (6–8). The first evidence for the importance of IL-21 in B cell differentiation was provided by studies carried out on IL-21R–deficient ($IL\text{-}21R^{-/-}$) mice (9). $IL\text{-}21R^{-/-}$ mice exhibited a severe defect in IgG1 production, whereas the secretion of IgE was augmented, both at a steady state and following immunization with T cell-dependent Ag. Using human EBV-infected B cell lines, we have demonstrated that IL-21–mediated signaling through the JAK/STAT pathway was required for the differentiation of B cells into late plasma blasts/early plasma cells (10). In mature B cells, binding of IL-21 to the IL-21R induces activation of STAT1, STAT3, and to a lesser extent STAT5 (10, 11).

Multiple studies have demonstrated that the effects of IL-21 stimulation of B cells depend on the cell signaling context (12–14). For instance, in the presence of signaling through both the BCR and CD40, IL-21 promotes growth and differentiation of murine splenic B cells into Ig-secreting cells (12, 13). Conversely, growth arrest and apoptosis occur following addition of IL-21 to B cells stimulated with LPS, CpG, or anti-IgM in the absence of T cell help (12, 14). These and other results have led to a consensus that the IL-21-IL-21R system is context dependent and plays an important role in maintaining B cell homeostasis. One consequence of a breakdown in this system could be impaired elimination of autoreactive B cells and the subsequent development of Ab-mediated autoimmune diseases like systemic lupus erythematosus (SLE). In this regard, lupus-prone BXSB.B6-Yaa^+/J mice showed elevated levels of serum IL-21 (13). In addition, a recent study has shown that BXSB-Yaa^+/J mice deficient for IL-21R failed to develop the disease (15).

To date, the majority of IL-21 studies have focused on its role in the final stage of B cell differentiation. However, IL-21R has also been reported to be expressed on early B cells progenitors in the BM (12), although it is not known whether it is functional and involved in the regulation of B cell development in the BM. One study reported that $IL\text{-}21R^{-/-}$ mice have normal numbers and phenotypes of B cells in the BM (9), although several specific populations of B cell progenitors, of interest to us, were not examined. Although this study shows that IL-21 does not have an essential, nonredundant role in B cell development, more recent data show that IL-21 can contribute to early hematopoiesis. It has

*Ontario Cancer Institute, Princess Margaret Hospital, University Health Network, Toronto, Ontario M5G 2M9, Canada; †Department of Immunology, University of Toronto, Toronto, Ontario M5S 1A8, Canada; ‡Laboratory of Molecular Immunology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892; and §Department of Medical Biophysics, University of Toronto, Toronto, Ontario M5G 2M9, Canada

[1]N.S. and D.K. contributed equally to this work.

Received for publication December 10, 2010. Accepted for publication February 23, 2011.

This work was supported by Grant 9862 from the Canadian Institutes of Health and Research and by the Terry Fox Foundation. N.S. received a doctoral award from the Canadian Institutes of Health and Research.

Address correspondence and reprint requests to Dr. Nathalie Simard, Ontario Cancer Institute, Princess Margaret Hospital, University Health Network, 610 University Avenue, No. 8-105, Toronto, Ontario M5G 2M9, Canada. E-mail address: n.simard@utoronto.ca

Abbreviations used in this article: BLIMP1, B lymphocyte-induced maturation protein 1; BM, bone marrow; CSR, class switch recombination; day $4^{IL\text{-}7}$, 4-d culture with IL-7; GLT, germline transcript; $IL\text{-}21R^{-/-}$, IL-21R–deficient; KSL, c-Kit^+sca^+ $lin^{-/low}$; SLE, systemic lupus erythematosus; Ta, annealing temperature; WT, wild-type.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1004040

been found that murine hematopoietic progenitor c-Kit$^+$sca$^+$lin$^{-/low}$ (KSL) cells express low levels of IL-21R (16). When grown in vitro with a mixture of c-Kit ligand, Flt-3L, and IL-7, the proliferation of KSL cells is enhanced in the presence of IL-21 (16). In addition, overexpression of IL-21 in vivo increased the number of KSL cells in the spleen (16). Another study showed that IL-21 did not have any mitogenic effect on total murine BM cells. However, apoptosis of BM CD11b$^-$ lymphoid cells that expressed IL-21R was delayed when the cells were cultured in the presence of IL-21 (17). On the basis of this information and the fact that very few B220$^-$ cells express IL-21R (12), the authors hypothesized that IL-21 acts mainly on lymphoid B220$^+$ B cell subsets in the BM. Finally, it has been reported that IL-21 transgenic mice have increased number of immature B cells in the spleen (13). One explanation for this phenotype could be increased maturation of BM B cell precursors. Collectively, these studies indicate that further investigation is required to determine the exact role of IL-21 in development of B cell progenitors in the BM.

In this study, we show that IL-21 message is constitutively expressed in murine BM CD4$^+$ T cells. IL-21R is expressed and is functional on all subsets of B cell progenitors, including pro-B, pre-B, and immature/mature B cells. In vitro culture of B cell progenitors with IL-21 is sufficient to induce expression of *Aid* and *Blimp1* and germline transcripts (GLTs) in these cells and to accelerate their development into mature B cells. A role for IL-21 in promoting B cell maturation is further supported by data that show an increase in the number of pro-B cells and a decrease in number of mature B cells in BM of IL-21R$^{-/-}$ mice. Finally, stimulation of the IL-21R and CD40 on B cell progenitors results in the formation of Ig-secreting cells.

Materials and Methods

Mice

Female C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) at 6–8 wk of age. IL-21R$^{-/-}$ mice were provided by Dr. W.J. Leonard (Laboratory of Molecular Immunology, National Hearth, Lung, and Blood Institute, National Institutes of Health). Mice were bred at the Ontario Cancer Institute and sacrificed according to a protocol approved by the animal care committee of the Ontario Cancer Institute.

Culture of BM B cell progenitors

Single-cell suspension from BM was prepared by crushing and gently grinding both femurs and tibiae using a mortar and pestle. Cells were pelleted and resuspended in RBC lysis buffer (150 mM NH_4Cl, 100 mM $NaHCO_3$, and 1 mM EDTA [pH 8]) for 1 min on ice. Recovered cells were filtered through a 40-μm cell strainer and cultured in freshly reconstituted OptiMEM supplemented with 10% non–heat-inactivated FCS, 50 μM 2-ME, 2.4 g/l $NaHCO_3$, and 100 μg/ml penicillin-streptomycin and grown at 37°C in a 5% CO_2 atmosphere. Supernatant from the stably transfected J558 line was used as a source of IL-7 (supplied by Dr. A. Cumano, Institut Pasteur, Paris, France). A total of 30 ng/ml IL-21 and/or 2 μg/ml anti-CD40 were added to the culture where indicated.

Isolation of BM B cell progenitors

BM cells were isolated by gently grinding both femurs and tibiae using a mortar and pestle. The cells were then flushed with MACS buffer ($PBS^{-Ca2+, -Mg2+}$, 1 mM EDTA, and 0.25% BSA). B cell progenitors were isolated directly using anti-B220 Ab coupled to beads (clone RA3-6B2; Miltenyi Biotec). A VarioMACS magnet with an LS adaptor was used to positively select the cells. Cells were washed three times with MACS buffer and eluted in OptiMEM (Life Technologies). Typically, 8–14 × 10^6 B220$^+$ cells are recovered from BM (two femurs and two tibiae) of a mouse.

Sorting of BM cells

B cell populations. B220$^+$ cells isolated by MACS were cultured with IL-7 for 4 d (day 4^{IL-7}) as described above. On day 4, cells were labeled with appropriate Abs and sorted using a FACSAria (BD Biosciences) to a greater than 98% purity. To stain pro-B (CD2$^-$κ/λ$^-$), pre-B (CD2$^+$κ/λ$^-$), and immature/mature (CD2$^+$κ/λ$^-$) BM B cells, rat anti-mouse CD2-PE (RM2-5; eBioscience), rat anti-mouse κ-FITC (Southern Biotechnology Associates), and rat anti-mouse λ-FITC (Southern Biotechnology Associates) were used.

Other populations. For experiments presented in Fig. 1, freshly isolated BM or BM B220$^-$ cells were stained with rat anti–mouse-B220-allophycocyanin (RA3-6B2), –CD3-FITC (145-2C11), –CD4-biotin (GK1.5), –CD8-PE (53-6.7), –CD44-allophycocyanin (IM7), –CD69-PE, and –NK1.1-allophycocyanin (PK136) (BD Biosciences). Cells were then sorted into CD4 T cells (CD3$^+$CD4$^+$NK1.1$^-$), CD8 T cells (CD3$^+$CD8$^+$ NK1.1$^-$), NK cells (NK1.1$^+$), and B cells (B220$^+$) (Fig. 1*B*). To sort naive CD4 T cells (CD3$^+$CD4$^+$CD44^{lo}CD69$^-$) and memory CD4 T cells (CD3$^+$ CD4$^+$CD44^{hi}CD69$^-$) (Fig. 1*C*), BM from 10 mice was first enriched for CD4$^+$ cells by negative selection using EasySep (StemCell Technologies) according to the manufacturer's instructions.

FACS analysis

Cells were washed with ice-cold PBS containing 3% FCS (v/v) and then incubated for 30 min on ice with predetermined concentration of FACS Abs in a total volume of 100 μl. The following Abs (clone) were used: IgM-biotin (33.60), B220-FITC, B220-allophycocyanin (RA3-6B2; eBioscience), CD19-allophycocyanin (MB19-1), CD2-PE (RM2-5; eBioscience), CD43-PE (S7-5; BD Biosciences), IgD-FITC (clone SBA.1; Southern Biotechnology Associates), IL-21R-biotin (eBio4A9; eBioscience), κ-FITC (Southern Biotechnology Associates), λ-FITC (Southern Biotechnology Associates), IgM (clone 33.60; made in-house), and rat IgG-biotin (R35-95; BD Pharmingen). For indirect staining, cells were washed twice after binding of the primary Ab and incubated with streptavidin-PerCP (BD Biosciences) for 15 min on ice. Samples were kept at 4°C in the dark and analyzed using a FACSCalibur (BD Biosciences). A total of 10,000 cellular events were analyzed for each sample.

Detection of IL-21 protein

BM cells from 10 mice were incubated in RBC lysis buffer (150 mM NH_4Cl, 100 mM $NaHCO_3$, and 1 mM EDTA [pH 8]) for 1 min on ice and washed in PBS/FCS. To isolate the CD4$^+$ population, BM cells were enriched for the CD4$^+$ T cell population by negative selection using EasySep (StemCell Technologies) according to the manufacturer's instructions and then labeled with an anti–CD4-PE Ab for sorting on a FACSAria (BD Biosciences). Sorted BM CD4$^+$ T cells were culture for 3 d with anti-CD3 (10 μg/ml) and anti-CD28 (2 μg/ml) with or without human IL-6 (100 ng/ml). On day 3, the supernatants were collected, and the presence of IL-21 was detected by cytometric bead array according to the manufacturer's protocol (BD Biosciences) with the following modification: 75 μl supernatant was added to 25 μl beads.

Real-time PCR and PCR

Total RNA was isolated from total BM of C57BL/6, Rag2$^{-/-}$, and TCRβ$^{-/-}$ mice or from BM B cell progenitors from C57BL/6 mice using the TRIzol reagent (Life Technologies) or RNeasy kit (Qiagen) according to the manufacturer's instructions. First-strand cDNA was prepared from 0.5 to 3 μg total RNA in a 20-μl reaction volume using the Superscript II (Life Technologies). After reverse transcription, *Il21*, *Blimp1*, and *Aid* were amplified by real-time PCR according to the manufacturer's instructions (Applied Biosystems). Amplification of actin was used for sample normalization. The following PCR primers were used: *Il21*, 5′-CGCCTCCTGATTAGACTTCG-3′ (sense) and 5′-TGGGTGTCCTTTTCTCATACG-3′ (antisense); *Blimp1*, 5′-TAGACTTCACCGATGAGGGG-3′ (sense) and 5′-GTATGCTGCCAACAACAGCA-3′ (antisense); *Aid*, 5′-GCGGACATTTTTGAAATGGTA-3′ (sense) and 5′-TTGGCCTAAGACTTTGAGGG-3′ (antisense); and *β-Actin*, 5′-GCCAACCGTGAAAAGATGACCCAG-3′ (sense) and 5′-ACGACCAGAGGCATACAGGGACAG-3′ (antisense).

Semiquantititative RT-PCR for *Il21r* and actin was performed on three serial dilutions of cDNA isolated from BM cells sorted into pro-B (CD2$^-$ LC$^-$), pre-B (CD2$^+$LC$^-$), and immature/mature (CD2$^+$LC$^+$) B cell populations. PCR products were amplified using the following conditions: for *Il21r*, annealing temperature (Ta) = 67°C, 34 cycles, and for *β-Actin*, Ta = 58°C, 22 cycles. Amplification of *β-Actin* was used as a cDNA loading control. The *β-Actin*–specific primers were 5′-TCCCTGGAGAAGAGCTACGA-3′ (sense) and 5′-ATCTGCTGGAAGGTGGACAG-3′ (antisense). Primers for *Il21r* were 5′-ATGCCCCGGGGCCCAGTGGCTG-3′ (sense) and 5′-CACAGCATAGGGGTCTCTGAGGTTC-3′ (antisense).

Class switch recombination

BM from C57BL/6 was cultured for 4 d in IL-7 and then sorted into pro-B (B220$^+$CD2$^-$κλ$^-$), pre-B (B220$^+$CD2$^+$κλ$^-$), and immature/mature (B220$^+$

$CD2^+\kappa\lambda^+$) B cells. Cells were then cultured without supplements (ctr), with IL-21 (30 ng/ml), with anti-CD40 (2 μg/ml), or with anti-CD40/IL-21 for 24 h prior to RNA extraction. After cDNA synthesis, samples were analyzed for class switch recombination (CSR). Primers for GLTγ2b were described previously (18). Amplification of GLTγ2b was done using the following conditions: Ta = 62°C, 40 cycles.

Western blot analysis

Sorted BM B cell progenitors or $B220^+$ BM cells day $4^{IL\text{-}7}$ were stimulated with 50 ng/ml IL-21 or IL-7 for 15 min and then lysed in 1% Nonidet P-40, 150 mM NaCl, 20 mM Tris-HCl (pH 7.4), 10 mM NaF, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 1 mM PMSF, and 5 μg/ml aprotinin and leupeptin (Roche) on ice for 30 min. Equal amounts of cell lysates were separated onto a 4–12% gradient NuPAGE gel and then transferred to a polyvinylidene difluoride membrane. Membranes were blocked with 5% milk in PBS/0.05% Tween/5% BSA (TBST) for 1 h at room temperature and then probed overnight at 4°C for pSTAT3, pSTAT1, pSTAT5 (Cell Signaling Technology), or actin (NeoMarkers). After several washes in TBST, membranes were subsequently probed with an HRP-coupled goat anti-rabbit IgG Ab or peroxidase-coupled goat anti-mouse IgG diluted 1/10,000 in TBST containing 5% milk for 45 min. Detection was performed using the ECL substrate (Amersham Biosciences) as described by the manufacturer.

ELISA

Enzyme immunoabsorbant plates (no. 3590; Costar) were coated with 5 μg/ml goat anti-mouse IgM (Jackson ImmunoResearch Laboratories), IgG1, IgG2a, IgG2b, IgG3, or IgA (Sigma-Aldrich) overnight at 4°C. Plates were washed with distilled water several times and blocked for 40 min at room temperature with 3% FCS/PBS. After washing, 50 μl culture supernatant was added, and plates were incubated at room temperature for 40 min. A standard curve was established using purified mouse IgM (BD Pharmingen), IgG1 κ (Sigma-Aldrich), IgG2a κ, IgG2b κ, IgG3 κ, and IgA λ isotype standards (BD Pharmingen). Plates were washed several times with distilled water. Plate-bound Abs were detected after a 40-min incubation with anti-mouse IgM, anti-mouse IgG, or anti-mouse IgA conjugated to peroxidase (Sigma-Aldrich). After washing, BD OptEIA substrate (BD Biosciences) was added to the plates, and the absorbance was read according to the manufacturer's instructions.

Statistical analysis

Statistical significance was assessed by two-tailed Student *t* test, and the level of significance was established at $p < 0.05$.

Results

IL-21 is expressed and secreted by $CD4^+$ T cells in BM

To determine whether B cell progenitors could encounter IL-21 during development in the BM, we performed RT-PCR on BM cells isolated from WT mice kept in pathogen-free conditions. Our results show that *Il21* mRNA was expressed in the total BM of WT mice (Fig. 1*A*). To further define the source of *Il21* message, we analyzed the BM of $RAG^{-/-}$ mice, which lack mature B and T cells but contain normal numbers of stromal cells and other hematopoietic cells, as well as the BM of $TCR\beta^{-/-}$ mice, which lack mature αβ T cells. We failed to detect the expression of *Il21* mRNA in either $RAG^{-/-}$ and $TCR\beta^{-/-}$ BM (Fig. 1*A*). These results suggest that *Il21* message is expressed by BM lymphocyte subsets, most likely T cells. To establish whether BM T cells produce *Il21*, we sorted different populations of BM cells including $CD4^+$ and $CD8^+$ T cells. We found expression of *Il21* mRNA only in the $CD4^+$ T cell population (Fig. 1*B*). To further characterize which subpopulation of T cells is producing *Il21*, we performed real-time PCR on sorted naive and memory BM T cells (Fig. 1*C*). We found that *Il21* mRNA was produced mainly by memory T cells.

It has been reported that IL-21 protein is secreted by splenic $CD4^+$ T cells activated with anti-CD3, -CD28, and anti–IL-6 (18). Therefore to determine whether IL-21 protein was secreted by BM $CD4^+$ T cells, we cultured BM $CD4^+$ T cells with anti-CD3, anti-CD28 with or without IL-6. Data collected by cytometric bead array assay show that CD3- and CD28-mediated signals were sufficient to induce IL-21 secretion by BM $CD4^+$ T cells (Fig. 1*D*). The greater number of cells observed in wells containing IL-6 likely explains the higher level of IL-21 detected in these wells.

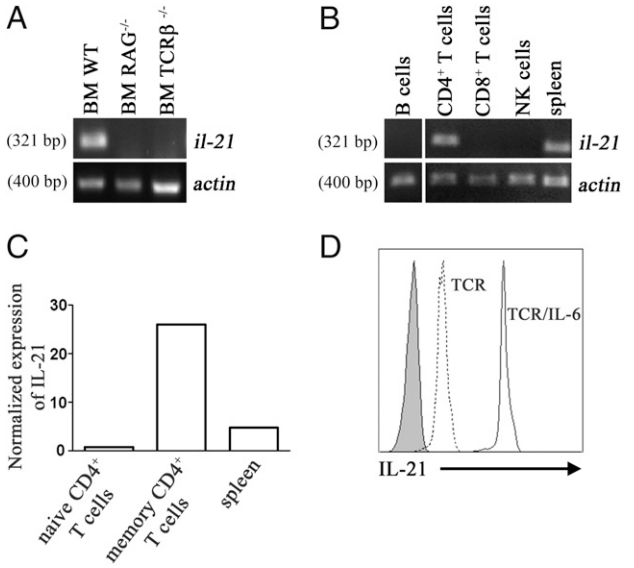

FIGURE 1. IL-21 is produced by $CD4^+$ T cells in BM. *A*, RT-PCR was performed on cDNA obtained from BM cells of WT, $Rag^{-/-}$, and $TCR\beta^{-/-}$ mice. *B*, Freshly isolated BM or BM $B220^-$ cells from WT mice were stained with anti-B220, -CD3, -CD4, -CD8, and -NK 1.1 Abs and sorted into B cells ($B220^+$), $CD4^+$ T cells ($B220^-CD3^+CD4^+NK1.1^-$), $CD8^+$ T cells ($B220^-CD3^+CD8^+NK1.1^-$), and NK cells ($B220^-NK1.1^+$). RNA was extracted, and RT-PCR was performed using primers specific for *Il21* and *Actin* as described in *Materials and Methods*. Spleen was used as a positive control. *C*, Freshly isolated BM cells from WT mice were stained with anti-CD3, -CD4, -CD44, and -CD69 and sorted into naive T cells ($CD3^+CD4^+CD44^{lo}CD69^-$) and memory T cells ($CD3^+CD4^+CD44^{hi}CD69^-$) populations. *Il21* mRNA expression was measured by real-time PCR and normalized to *β-Actin*. Spleen was used as a positive control. The figure is a representative experiment of two independent experiments. *D*, BM $CD4^+$ cells were sorted and cultured with anti-CD3 (10 μg/ml) and anti-CD28 (2 μg/ml) with IL-6 (100 ng/ml) (solid line) and without IL-6 (dashed line). After 72 h, IL-21 protein was detected in the supernatant by FACS with beads array. Data presented are a representative experiment that was performed two times with similar results.

IL-21R is expressed on BM B cell progenitors

The developmental stages of B lymphopoiesis can be characterized by different profiles of cell surface proteins. We have previously shown that CD2 and μ H chain, or CD2 and κ + λ L chains, constitute reliable developmental markers to follow the progression of progenitors from pro-B to pre-B to immature and mature B cell stages in in vitro culture (19). To analyze the expression of IL-21R on different BM B cell subsets, $B220^+$ BM cells were isolated and grown in day $4^{IL\text{-}7}$. We used a combination of Abs that recognize CD2 κ and λ L chain (anti-L chain) to select different B cell populations, including $CD2^-LC^-$ (pro-B), $CD2^+LC^-$ (pre-B), and $CD2^-LC^+$ (immature/mature B cells). Semi-quantitative RT-PCR results show that *Il21r* mRNA was present at low levels in pro-B cells and at higher levels in pre-B and immature/mature B cells (Fig. 2*A*). As expected, *Il21r* mRNA was not expressed in S17 stromal cells but was expressed in total spleen cells (Fig. 2*A*). Next, FACS analysis was performed on day $4^{IL\text{-}7}B220^+$ BM cells isolated from WT and $IL\text{-}21R^{-/-}$ mice to determine the expression of IL-21R on the cell surface of B cell progenitors. We found that IL-21R expression progressively increased with the developmental stage of B cell progenitors. IL-

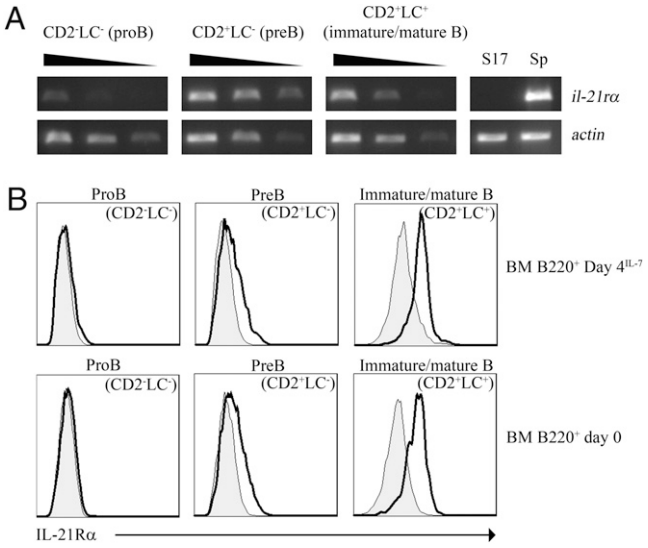

FIGURE 2. IL-21R expression on BM B cell progenitors. *A*, Day $4^{IL\text{-}7}$ $B220^+$ BM cells were stained with anti-CD2, -κ, and -λ Abs and sorted into pro-B ($CD2^-LC^-$), pre-B ($CD2^+LC^-$), and immature/mature ($CD2^+LC^+$) B cell populations. RNA was extracted, and semiquantitative RT-PCR was performed on serial dilutions of cDNA (undiluted, 1/5 dilution, and 1/10 dilution) using primers specific for IL-21R and actin as described in the *Materials and Methods*. The stromal cell line S17 was used as negative control, and spleen (Sp) cells were used as positive control. Semiquantitative RT-PCR was performed on four independent sample sets with similar results. *B*, Surface expression of IL-21R on B cell progenitors. *Top panels*, Day $4^{IL\text{-}7}B220^+$ BM cells from WT mice and IL-$21R^{-/-}$ mice were stained with anti-B220, -CD2, -κ LC, -λ LC, and –IL-21Rα. *Bottom panels*, Freshly isolated BM cells from WT mice and IL-$21R^{-/-}$ mice were stained with anti-CD2, -κ, -λ, and –IL-21Rα Abs and analyzed by FACS. Open and closed histograms represent binding of anti-mouse IL-21Rα Ab in WT and IL-$21R^{-/-}$ mice, respectively. Data are representative of two independent experiments. LC, L chain.

21R was below detection on $CD2^-LC^-$ pro-B cells, but it was expressed at low levels on $CD2^+LC^-$ pre-B cells and at high levels on $CD2^+LC^+$ immature/mature B cells (Fig. 2*B*, *upper panels*). This pattern of expression closely matched the expression pattern on freshly isolated BM B cells stained with the same markers (Fig. *2B*, *lower panels*).

IL-21 induces tyrosine phosphorylation of STAT1, STAT3, and STAT5 in B cell progenitors

To determine whether IL-21R was functional, we carried out signaling experiments on different subsets of B cell progenitors. Day $4^{IL\text{-}7}$ $B220^+$ cells were sorted as described above. $CD2^-LC^-$ pro-B cells, $CD2^+LC^-$ pre-B cells, and $CD2^+LC^+$ immature/mature B cells were stimulated with IL-21 (50 ng/ml for 15 min). Western blot analysis shows that the tyrosine phosphorylation of STAT3, STAT1, and STAT5 was induced after stimulation in all B cell populations (Fig. 3*A*). Moreover, the intensity of the signal correlated with the expression levels of IL-21R in the different B cell populations. It was the weakest in pro-B cells and the strongest in immature/mature B cells (Fig. 2*B*). To rule out the possibility that contaminating pre-B cells could account for the phosphorylation of STAT3 observed in the pro-B cell fraction, we spiked an IL-21–unresponsive B progenitor cell line with different numbers of sorted pre-B cells. We then measured the phosphorylation of STAT3 following IL-21 stimulation. Fig. 3*B* shows that contamination with pre-B cells >10% was necessary to achieve the level of STAT3 phosphorylation observed in IL-21–stimulated

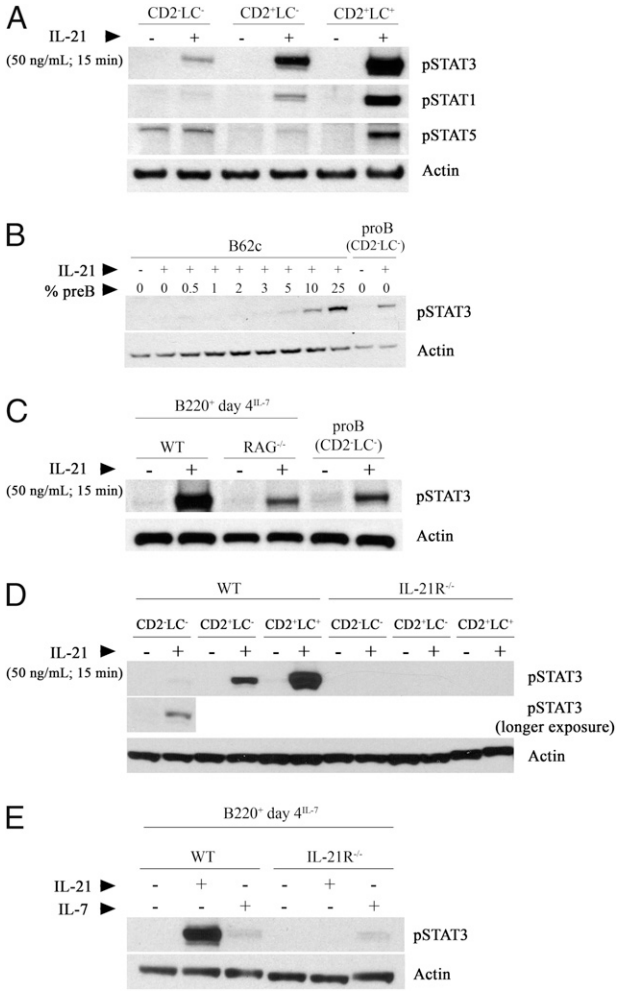

FIGURE 3. IL-21R stimulation increases tyrosine phosphorylation of STAT1, STAT3, and STAT5 in pro-B, pre-B, and immature/mature B cells. Day $4^{IL\text{-}7}B220^+$ BM cells from WT mice were sorted for pro-B ($CD2^-LC^-$), pre-B ($CD2^+LC^-$), and immature/mature B ($CD2^+LC^+$) cells. *A*, Tyrosine-phosphorylated forms of STAT1 (pY701), STAT3 (pY705), and STAT5 (pY694) were detected by Western blotting. Actin represents loading control. *B*, The IL-21–nonresponsive B62c pro-B cell line was spiked with different numbers of sorted pre-B cells, stimulated for 15 min with IL-21 (50 ng/ml), and analyzed for STAT3 phosphorylation by Western blotting. *C*, $B220^+$ BM cells were isolated from $RAG^{-/-}$ mice and grown in IL-7. On day 4, cells were harvested and stimulated for 15 min with IL-21 (50 ng/ml). Phosphorylation of STAT3 was detected by Western blotting. *D*, Day $4^{IL\text{-}7}$BM $B220^+$ cells from WT and IL-$21R^{-/-}$ mice were sorted into pro-B ($CD2^-LC^-$), pre-B ($CD2^+LC^-$), and immature/mature B ($CD2^+LC^+$) populations. Phosphorylation of STAT3 was detected by Western blotting. *E*, Day $4^{IL\text{-}7}$BM $B220^+$ cells from WT and IL-$21R^{-/-}$ mice were simulated with IL-7 or IL-21. Phospho-STAT3 was detected by Western blotting. Data are representative of two independent experiments. LC, L chain.

pro-B cells. Because the purity of the sorted pro-B cell fraction was 99.98%, this result indicated that the source of the phosphorylated STAT3 was an IL-21–stimulated pro-B cell and not a contaminating pre-B cell. To further test this finding, we isolated $B220^+$ BM cells from $RAG^{-/-}$ mice, which have a B cell developmental block at the pro-B cell stage. We found that IL-21 treatment of day$4^{IL\text{-}7}RAG^{-/-}B220^+$ BM cells activated phosphorylation of STAT3 (Fig. 3*C*). To exclude the possibility that a receptor other than the IL-21R was involved in the activation of STAT3, we stimulated pro-B, pre-B, and immature/mature B cells from WT and IL-$21R^{-/-}$ mice with IL-21. Our results show that

phosporylation of STAT3 occurred only in WT cells (Fig. 3*D*). Finally, we showed that phosphorylation of STAT3 could be induced by IL-7 but not by IL-21 in IL-21R$^{-/-}$ cells, indicating that STAT3 signaling is functional in IL-21R$^{-/-}$ cells (Fig. 3*E*). Taken together, these results clearly indicate that the IL-21R expressed on the surface of pro-B, pre-B, and immature/mature B cells is functional and that its stimulation can result in the phosphorylation of downstream proteins.

IL-21 accelerates the transition of pro-B cells toward the pre-B cell stage and the transition of pre-B cells toward the immature/mature B cell stage

To examine whether IL-21–mediated signals affect development of B cell progenitors, day 4$^{IL\text{-}7}$BM B220$^+$ cells isolated from WT and IL-21R$^{-/-}$ mice were sorted into pro-B, pre-B, and immature/mature B cell populations as previously described and cultured with or without IL-21 for 48 h. Absolute numbers of viable cells for each population were determined by using trypan blue exclusion. No significant difference in total cell numbers was observed between the control and IL-21–containing cultures (data not shown). However, FACS analysis showed that IL-21 treatment affected the percentages of maturing cells that arose from WT pro-B and WT pre-B cultures. In the cultures initiated from CD2$^-$LC$^-$ pro-B cells, there were more pre-B cells and fewer pro-B cells in the presence of IL-21 after 48 h (Fig. 4*A*, 4*B*). Similarly, we observed a trend toward increased percentages of immature/mature B cells and fewer pre-B cells in cultures initiated from CD2$^+$LC$^-$ pre-B cells in the presence of IL-21 (Fig. 4*C*, *top panel*, 4*D*, *top panel*). Further analysis showed that the percentage of immature/mature B cells expressing high levels of L chain tended to be higher in the IL-21–containing wells than in the control wells (Fig. 4*C*, *top panel*, CD2$^+$LC^{hi} population, and 4*D*, *lower panel*). We hypothesized that this population (CD2$^+$LC^{hi}) likely represents cells that express both IgM and IgD on the surface. In agreement with this, FACS analysis showed that the percentage of IgM$^+$IgD$^+$ mature B cells was higher in cultures grown with IL-21 than the percentage observed in the control cultures (Fig. 4*C*, *lower panel*). In support of the finding that the increase in percentages of maturing B cells is IL-21 mediated, we did not observe an increase in B cell development with IL-21 treatment in cultures initiated with IL-21R$^{-/-}$ cells (Fig. 4). Taken together, these results show that IL-21 accelerates the transition of pro-B toward the pre-B cell stage and of pre-B cells toward the mature B cell stage.

To determine whether IL-21 affects B cell development in a similar way in vivo, we performed FACS analysis on BM B cell progenitors freshly isolated from WT and IL-21R$^{-/-}$ mice. In agreement with the in vitro analysis, we found that IL-21R$^{-/-}$ mice have increased proportion of B220$^+$CD2$^-$IgM$^-$ pro-B cells ($p = 0.0376$) and smaller proportion of B220$^+$CD2$^+$IgM$^+$ immature/mature B cells ($p = 0.0131$) than WT mice (Fig. 5*A*, *top panel*). We confirmed this result using a different set of B cell development markers and found that both the percentages and absolute numbers of B220^{lo}CD43^{hi}IgM$^-$ pro-B were increased ($p < 0.0001$ and $p = 0.0021$, respectively), and the percentages and absolute numbers of B220^{hi}CD43$^-$IgM$^+$ mature B cells were decreased ($p = 0.0232$ and $p = 0.0756$, respectively) in IL-21R$^{-/-}$ (Fig. 5*A*, 5*B*, *middle panel*). The decrease in IgM$^+$ cells was due to a decrease in the IgM$^+$IgD^{hi} mature B cell population, more specifically in the IgM^{hi}IgD^{hi} cell population (percentage, $p = 0.0355$; absolute number, $p = 0.0053$) (Fig. 5*A*, 5*B*, *bottom panel*). These results confirm that IL-21 has an impact on B cell development in the BM and support the hypothesis that IL-21 accelerates the maturation of B cell progenitors.

IL-21 regulates the expression of Blimp1 and Aid, and induces the expression of GLTγ2b in B cell progenitors

In mature B cells, stimulation of the IL-21R has been shown to induce the expression of B lymphocyte-induced maturation protein 1 (BLIMP1) (13, 20). BLIMP1 is a DNA-binding zinc finger protein that can associate with certain methyl transferases and is important in the regulation of plasma cell differentiation (21). IL-21 also induces the expression of AID, an enzyme essential for CSR and somatic hypermutation, when costimulated with anti-CD40 or anti-CD40 and anti-IgM (22–24). Although no evidence of somatic hypermutation has been reported, CSR to IgG and IgA has been observed in human cord blood and mature B cells in response to IL-21 and anti-CD40 (22, 24, 25). Because BLIMP1 and AID are typically expressed by the peripheral B cells, it was of interest to determine whether IL-21 can activate a similar pattern of genes in B cell progenitors from the BM. For this purpose, day 4$^{IL\text{-}7}$B220$^+$ BM cells were stimulated with IL-21 for 24 h prior to being sorted into pro-B, pre-B, and immature/mature B cells. Real-time PCR results showed that IL-21 increased expression of *Blimp1* in pre-B cells (Fig. 6*A*). In contrast, we did not observe any changes in *Blimp1* expression in pro-B or immature/mature B cells. In addition, IL-21 clearly induced *Aid* expression in pre-B cells and immature/mature cells, but no significant effect was observed in the cultures initiated with pro-B cells (Fig. 6*B*).

In mature B cells, IL-21–induced expression of *Aid* has been associated with the initiation of CSR (25). CSR begins with the initiation of transcription of the GLTs from the promoter of a specific isotype. Therefore, we measured this early hallmark of CSR by RT-PCR. We searched for GLTs in unstimulated B cells and B cells stimulated with anti-CD40, IL-21, or anti-CD40 and IL-21. For this purpose, day 4$^{IL\text{-}7}$B220$^+$ BM cells were sorted into pro-B, pre-B, and immature/mature B cells and then stimulated for 24 h. Interestingly, IL-21 treatment of sorted pre-B and sorted immature/mature B cells resulted in increased levels of GLTγ2b compared with the untreated cells (Fig. 7*A*). Given that B cell progenitors continuously mature when cultured in vitro, we wanted to determine whether IL-21 exposure resulted in increased transcription of GLTs in the pre-B cells themselves or in immature/mature B cells that develop from pre-B cells. We treated bulk BM B220$^+$ day 3$^{IL\text{-}7}$ cultures with IL-21 for 24 h and then sorted the pro-B, pre-B, and immature/mature B population. Although there was a trend for increased GLT2γb in IL-21–treated cells, we did not observe a significant difference between stimulated versus unstimulated cells in progenitor populations that were treated with IL-21 prior to sorting (data not shown). Under these conditions, we also examined later hallmarks of CSR and did not observe IL-21–induced differences in postswitch transcripts and circular transcripts (data not shown). Collectively, our data show that IL-21 induces *aid* in pre-B cells but suggest that IL-21–initiated CSR occurs at later stages of B cell development.

Ig-secreting cells are generated from B cell progenitors stimulated with IL-21 and anti-CD40

It is known that IL-21 induces plasma cell differentiation and Ig secretion of human cord blood and CD19$^+$ peripheral blood and splenic B cells when used in combination with anti-CD40 (22, 24). IL-21–mediated induction of *Blimp1*, *Aid*, and GLTs described above is consistent with the hypothesis that IL-21 can drive the differentiation of BM B cell progenitors into Ig-secreting cells. To test this, we plated day 4$^{IL\text{-}7}$B220$^+$ BM cells with IL-7 and a combination of IL-21 and anti-CD40. In this experiment, IL-7 was included in the culture to allow for continued expansion of a pro-B

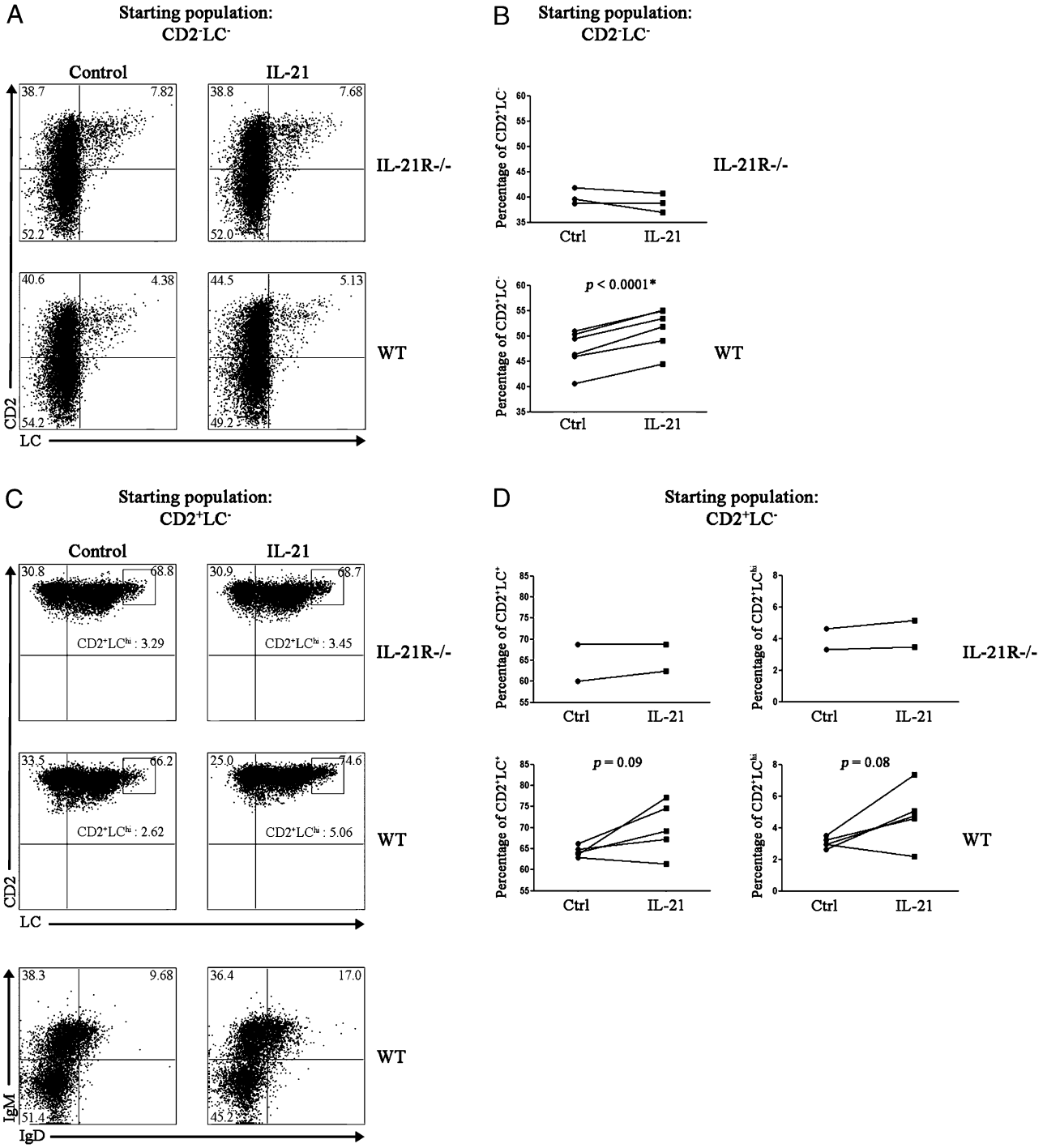

FIGURE 4. IL-21 regulates maturation of different B cell progenitors. Day $4^{IL\text{-}7}B220^+$ BM cells from WT and IL-$21R^{-/-}$ mice were sorted into pro-B ($CD2^-LC^-$), pre-B ($CD2^+LC^-$), and immature/mature B $CD2^+LC^+$) cell populations. Each population was plated with or without IL-21 (30 ng/ml) for 48 h. Control and IL-21–containing cell cultures initiated with pro-B (*A*, *B*) and pre-B (*C*, *D*) cells from IL-$21R^{-/-}$ and WT mice were harvested on day 2 of culture and analyzed by FACS using CD2-LC and IgM-IgD Ab combination. *A* and *C*, Percentages of cells in each population are shown on the plots of a representative experiment. *B* and *D*, Scatter plot of cell population percentages from separate experiments. Lines connect pairs of experiments performed. Statistical analysis of percentage of pre-B and immature/mature B cells obtained in control and IL-21–treated WT cell cultures was assessed by two-tailed paired Student *t* tests. The analysis shows that there were a significant increase percentage of pre-B cells in IL-21–containing culture started with pro-B cells compared with control (*B*, *lower panel*, $*p < 0.0001$) and a trend toward an increase percentage of immature/mature B cells in IL-21–containing culture initiated with pre-B cells (*D*, *lower left panel*, percentage of $CD2^+LC^+$, $p = 0.09$; *D*, *lower right panel*, percentage of $CD2^+LC^{hi}$, $p = 0.08$). LC, L chain.

subset, which kept maturing and replenishing pre-B and immature/mature subsets. This extended the survival of the overall cell culture, thereby giving it sufficient time to respond to IL-21 and anti-CD40. ELISAs were performed on supernatants collected on day 7 of culture. Fig. 7*B*, *left panel*, shows that both IL-21 and anti-CD40 were required to induce secretion by $B220^+$ cells. In these cultures, we detected mostly IgM and IgG3 but also IgG1 and IgG2b and some IgG2a Igs. However, we did not detect IgA (data not shown).

To determine whether early B cell progenitors can differentiate into Ig-producing cells, we sorted day $4^{IL\text{-}7}B220^+$ BM cells into pro-B cells. Pro-B cells were then cultured with IL-7 and anti-CD40 with or without IL-21. ELISAs were performed on super-

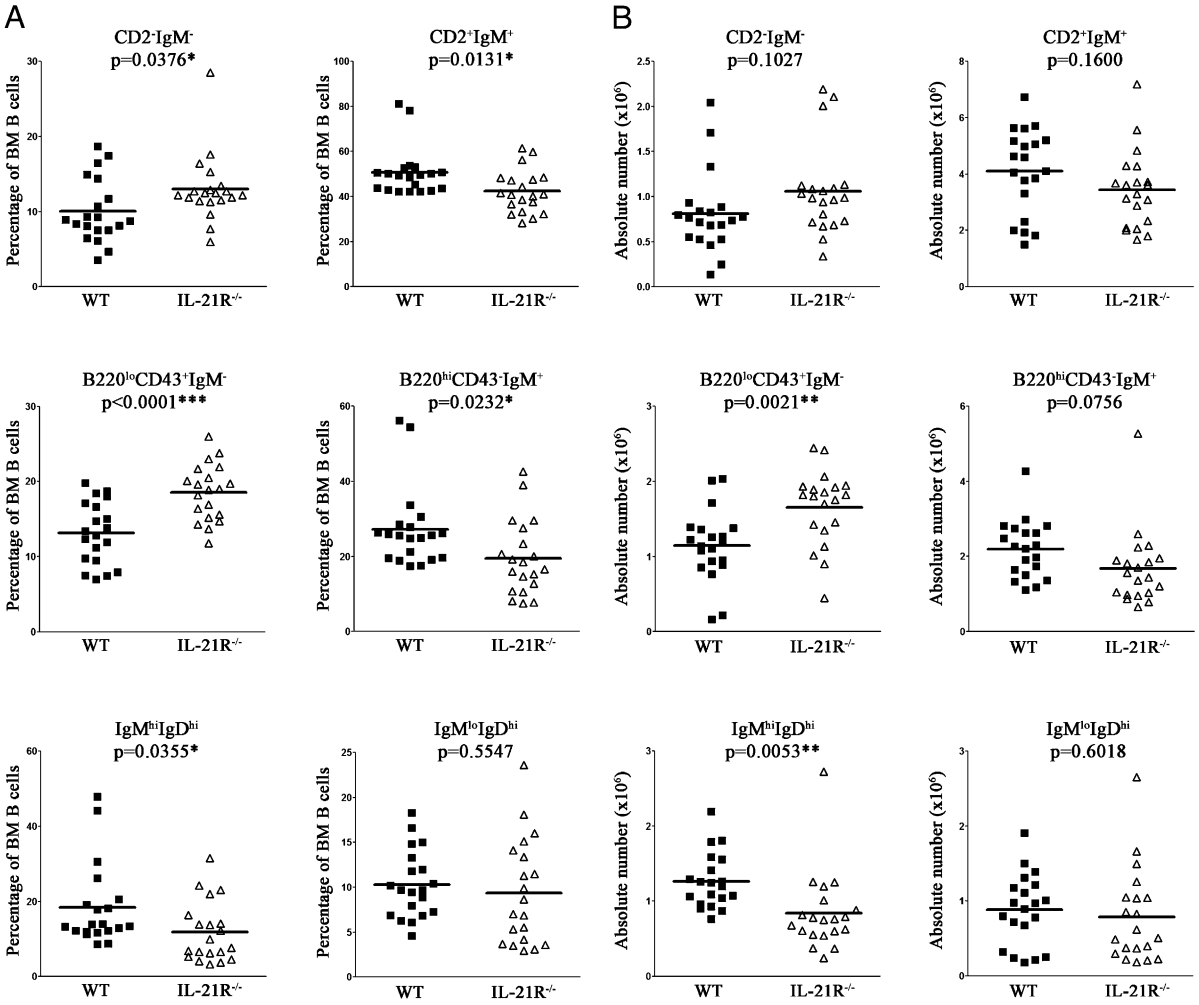

FIGURE 5. IL-21R$^{-/-}$ mice have more pro-B and fewer mature B cells than WT mice in BM. BM cells were isolated from WT ($n = 20$) and IL-21R$^{-/-}$ ($n = 20$) mice and stained for B220, CD43, and IgM or CD19, CD2, and IgM, or CD19, IgM, and IgD. Percentages (*A*) and absolute numbers (*B*) are shown for each cell population. Statistical significance was assessed by two-tailed Student *t* test, and the level of significance was established at $p < 0.05$.

natants harvested on day 9 of culture. We show that IL-21 and CD40 signals induced differentiation of pro-B cells into cells that secreted mostly IgG3 but also IgM, IgG1, IgG2a, and IgG2b in the presence of IL-21 and anti-CD40. IgA was below detection level of our assay (Fig. 7*B*, *right panel*).

Discussion

It is well established that IL-21 strongly influences the differentiation of murine and human B cells. To date, this has been demonstrated at the end stages of B cell development where IL-21 induces the transition from fully mature B cells to Ig-secreting plasma cells. In this report, we significantly extend the role of IL-21 by showing that IL-21 accelerates the maturation of murine B cell progenitors. Such cells are induced to express *Blimp1* and *Aid*, genes normally expressed in peripheral mature B cells. In addition, we show that IL-21 induces the first step of CSR in B cell progenitors by inducing GLTs and, together with anti-CD40, enables cells to differentiate into Ig-secreting cells.

Careful analysis of expression of IL-21R on different BM B cell progenitors revealed a gradual increase of cell surface IL-21R from pro-B cells to immature/mature B cells, consistent with the previous observations reported by Jin et al. (12). We extend these findings by showing that IL-21R is functional on these populations of B cell progenitors. The intensity of IL-21–induced STAT phosphorylation signals correlated with the level of IL-21R expression. The signals were the weakest in pro-B cells and the strongest in the immature/mature B cells. Even though we and others (12) failed to detect IL-21R protein at the surface of pro-B cells by FACS, the results from both RT-PCR and functional signaling experiments clearly show that IL-21R is also expressed on these cells. Given the unexpected nature of this finding, it was important to ensure that the observation was not based on contaminating pre-B cells. To examine this question, we used an IL-21–unresponsive pro-B cell line to which we added different numbers of FACS-sorted IL-21–responsive pre-B cells. We found that a contamination level of at least 10% pre-B was required to attain the level of STAT3 phosphorylation observed in sorted pro-B cells stimulated with IL-21. This threshold is much higher than the 0.5–1% contamination levels we routinely achieve when we enrich for pro-B cells. Furthermore, we found that IL-21 stimulation of BM B cells isolated from RAG$^{-/-}$ mice, where B cell development is blocked at the pro-B cell stage, also induces phosphorylation of STAT3. Finally, IL-21 stimulation failed to induce phoshorylation of STAT3 in IL-21R–deficient pro-B cells, confirming that the phosphorylation of STAT3 in IL-21–stimulated pro-B cells occurs through the IL-21R.

We also found that *Il21* message is constitutively expressed in murine BM, providing evidence that B cell progenitors may well encounter IL-21 during development. Activated Th17, T follicular

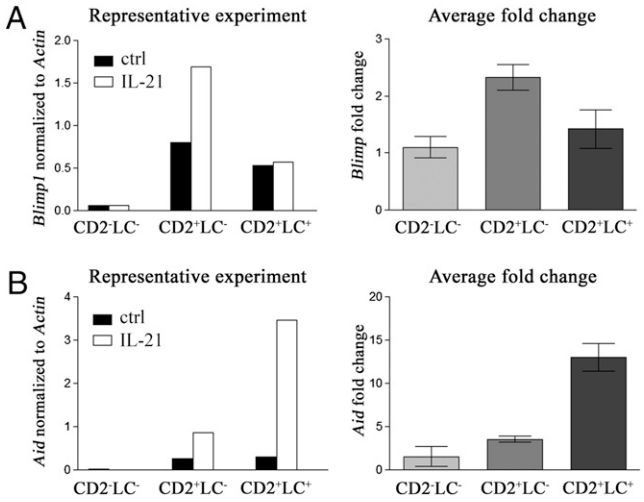

FIGURE 6. IL-21 regulates gene expression of *Blimp1* and *Aid* in B cell progenitors. B220$^+$ BM cells were grown in IL-7. On day 3, IL-21 (30 ng/ml) was added for 24 h to half of the culture. Cells were sorted on day 4 into pro-B (CD2$^-$LC$^-$), pre-B (CD2$^+$LC$^-$), and immature/mature B (CD2$^+$LC$^+$) cells, and total RNA was extracted. Real-time PCR analysis using *Blimp1* (*A*)- and *Aid* (*B*)-specific primers were performed on cDNA as described in *Materials and Methods*. Figures show normalized values from a single experiment (*left panel*) and fold change over untreated from two independent experiments (*right panel*). LC, L chain.

helper, and NKT cells are thought to be the main sources of IL-21 in the periphery (26–29). Consistent with a T cell origin of the *Il21* transcript detected in the BM, we detected *Il21* message specifically in CD4$^+$ T cells. Furthermore, we showed that BM CD4$^+$ T cells require fewer stimuli to secrete IL-21 protein than splenic CD4$^+$ T cells. In contrast to splenic naive and memory CD4$^+$ T cells, which require stimulation through CD3, CD28, and IL-6R (18), IL-6R stimulation was not required to induce secretion of IL-21 protein in BM CD4$^+$ T cells.

One of the main findings of our study is that IL-21 promotes the development of B cell progenitors. This is supported by in vivo data showing that IL-21R$^{-/-}$ mice have more pro-B cells, which is likely a consequence of slower maturation. These mice also have fewer mature B cells in their BM than WT mice, although based on current models (30–32), we cannot distinguish whether this difference in IgM^{hi}IgD^{hi} cells comes from the newly arising B cells or from the recirculating B cells.

In further support of our in vivo observation, in vitro stimulation of sorted pro-B and pre-B progenitors by IL-21 accelerates their development. The increased percentage of B cells at a more advanced developmental stage is unlikely to be a consequence of enhanced proliferation or survival in response to IL-21. This is supported by the finding that the total number of cells recovered after 48 h of culture is similar between IL-21–treated and control wells. Moreover, after 48 h of treatment with IL-21, annexin V and 7-aminoactinomycin D staining showed variable cell survival with no observable trend in cultures initiated with pro-B or pre-B cells (data not shown). It should be noted that although the experiments reported in this paper show an effect of IL-21 on purified B cell progenitors in tissue culture, it may be the case that other factors are involved in vivo. For example, it has been shown that IL-21 synergizes with Flt3L and IL-15 to increase proliferation and promote differentiation of NK cells from BM progenitors (33).

Several lines of evidence show that IL-21 can induce *Blimp1* and, when used in combination with anti-CD40 or anti-IgM, IL-21

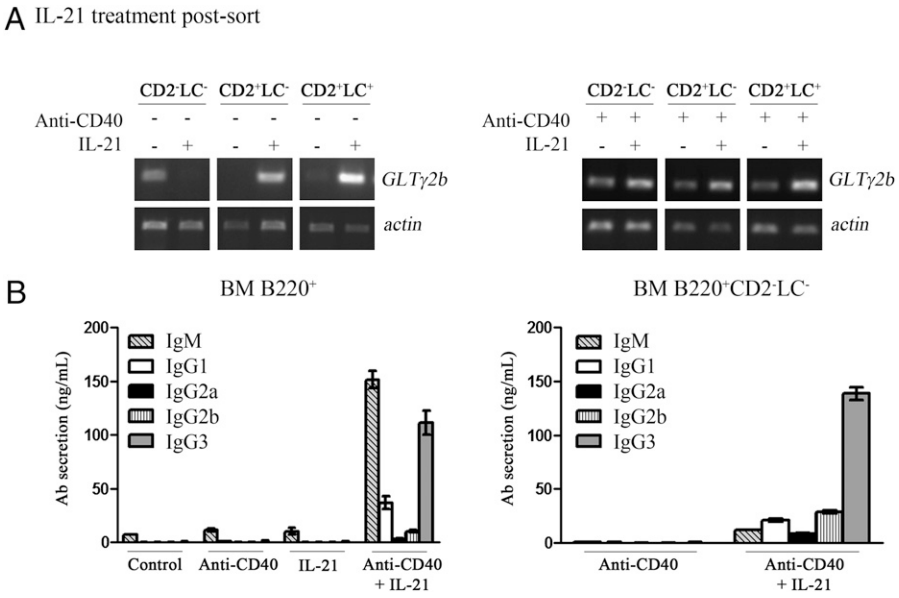

FIGURE 7. IL-21R stimulation induces the expression of GLT γ2b. *A*, Day 4^{IL-7} BM cells were sorted into pro-B (B220$^+$CD2$^-$LC$^-$), pre-B (B220$^+$CD2$^+$LC$^-$), and immature/mature B (B220$^+$CD2$^+$LC$^+$) cells. Each population was plated with IL-21 (30 ng/ml), anti-CD40 (2 μg/ml) or anti-CD40 and IL-21. After 24 h, total RNA was extracted, and RT-PCR analysis was performed to detect GLTγ2b. *β-Actin* was used to normalize for cDNA input. Band intensity was measured and normalized to *β-Actin* using Quantity One software. The figure shows one representative experiment that has been performed at least three times with similar results. In pre-B cells stimulated with IL-21 only, the fold increase in GLT levels ranged from 1.12- to 3.11-fold; in immature/mature B cells, the fold increase ranged from 1.23 to 6.25. Statistical analysis of quantitated and normalized band intensity for pre-B cells was performed using the Wilcoxon test demonstrating that there was a significant fold increase with IL-21 treatment in pre-B (CD2$^+$LC$^-$)-sorted cells ($p = 0.032$; $n = 6$). *B*, IL-21 in combination with anti-CD40 induces the formation of Ig-secreting cells from cultures initiated from B cells progenitors. *Left panel*, Day 4^{IL-7} B220$^+$ BM cells were plated with IL-7 in combination with anti-CD40, IL-21, or both. Supernatants were harvested on day 7, and ELISA was performed as described in *Materials and Methods*. *Right panel*, Day 4^{IL-7}B220$^+$ BM cells were sorted into pro-B cells (CD2$^-$LC$^-$) and plated with IL-7 and anti-CD40 with or without IL-21. Culture supernatants were collected on day 9, and ELISA was performed as described in *Materials and Methods*. Results are representative of three independent experiments. LC, L chain.

can induce *Aid* in different populations of mature B cells (13, 22–24). We show that IL-21 increases expression of *Blimp1* in developing B cells, and in particular, in pre-B cells. It is possible that the increase in *Blimp1* expression contributes to the acceleration of maturation of pre-B cell progenitors observed in presence of IL-21 by inducing genes normally found in mature B cells and repressing genes associated with early B cell progenitors. We also show that IL-21 alone induces *Aid* as early as the pre-B cell stage. This result contrasts with data obtained on other type of B cells where anti-CD40 or anti-CD40 and anti-IgM are required. Although this is not the first report of early B lineage cells expressing *Aid* and *Blimp1* (34–36), to our knowledge, it is the first study to suggest a method of induction in early B cell progenitors.

Importantly, our study shows that IL-21 alone was sufficient to initiate early steps of CSR in BM B cell progenitors. This is in contrast to what has been reported for human cord blood B cells in which both IL-21 and anti-CD40 are required for GLTs to occur (24, 25). However, similar effects on GLTs have been observed in bulk human $CD19^+$ spleen cells, even though *Aid* expression was absent (24). Although expression and function of *Aid* is generally associated with the germinal center reaction in the secondary lymphoid organs, a recent study has shown that *aid* message is expressed in early pro-B/pre-B cell progenitors in vivo and is responsible for the CSR observed in these cells (34). Moreover, other studies have shown that CSR can occur in some of the Abelson-transformed pre-B cell lines (37–39).

We believe it is highly unlikely that IL-21R, *Aid*, *Blimp1*, GLTγ2b messages come from the contaminating BM plasma cells and BM memory B cells. First, BM B cells used in our study were selected using an anti-B220 Ab, a molecule absent from BM plasma cells (40). Second, we used anti-κ and anti-λ Abs instead of anti-μ to negatively select pro-B and pre-B cells and thereby avoid possible contamination of these populations with memory cells expressing IgM or any other H chain isotype.

Our results show that pro-B cells differentiate into plasma cells secreting IgM, IgG1, IgG2a, IgG2b, or IgG3 Igs when costimulated with IL-21 and anti-CD40. Seagal et al. (41) have proposed that isotype-switched B cell precursors are deleted under normal physiological conditions by a mechanism involving Fas/FasL interaction, presumably to prevent autoimmunity. However, presence of IgA and IgG in μMT mice (42, 43) suggests that at least some isotype-switched cells can bypass this mechanism. Two hypotheses have been proposed to explain the presence of CSR in BM B cell progenitors. One hypothesis is that these cells could be the product of an alternative B cell development pathway (43). Alternatively, signals through BCR and TLRs can induce *aid* expression, which is involved in CSR observed in some B cell progenitors. Class-switched B cell progenitors could be responsible for enhanced innate immunity by generating IgG- or IgA-producing cells (34).

Having demonstrated that developing B cell progenitors express IL-21R and show accelerated maturation in response to IL-21, it is of critical importance to determine what role the IL-21/IL-21R pathway plays in the life of a B cell. One possibility is that T cell-derived IL-21 contributes to the "normal" development of B cells in the BM. $CD8^+$ and $CD4^+$ mature T cells constitute ~3–8% of total BM-nucleated cells (44). Several studies reported that, at a steady state, most $CD4^+$ T cells in murine BM have an activated phenotype (45, 46). Maintenance of the activation state of BM T cells does not require Ag stimulation but occurs in response to factors produced by the local microenvironment, such as IL-7, IL-15, and 4-1BBL (44, 46–49). There is evidence for interaction between BM T cells and BM B cells through CD40/CD40L that would be required for maintaining bone homeostasis (50). In addition, it has been reported that hematopoiesis is severely impaired in T cell-deficient nude mice and that restoration of the BM $CD4^+$ T cells rescued normal development of the myeloid compartment (46). Our discovery that IL-21 in the murine BM is produced by T cells reinforces the potential importance of a T cell-dependent B cell developmental pathway option.

An alternative, but not mutually exclusive, interpretation is that IL-21 influences B cell development in the context of an inflammatory response. It is known that leukocyte production is affected during infection and inflammation. Experimental inflammation caused by the injection of adjuvants leads to a remarkable decline in BM B cell development and a corresponding onset of extramedulary development in the spleen (51). This phenomenon is mediated by a rapid reduction of CXCL12, which is thought to be involved in the sequestration of developing B cell progenitors in the BM (51). Under these conditions, B cell progenitors might be found in areas with active $CD4^+$ T cells in the spleen. Interaction of B cell progenitors with T cells through CD40–CD40L interaction in the presence of IL-21 could allow B cell progenitors that have been mobilized to the periphery to continue their development outside the BM and participate in the immune response. It has been noted that such extramedulary development might bypass checkpoints that normally eliminate autoreactive cells in the BM (52). Indeed, there is a growing body of evidence linking IL-21 with the development of some autoimmune diseases with strong humoral component. For example, BXSB.B6-Yaa^+/J murine model of SLE shows elevated levels of serum IL-21 (13). In contrast, BXSB-Yaa^+ mice deficient for IL-21R do not develop SLE (15).

We do not yet know whether our observations have uncovered a "normal" process that may contribute to the immune response or even a "dangerous" anomaly that may contribute to autoimmunity by allowing immature cells to bypass regulatory mechanisms that normally eliminate autoreactive cells. However, our results clearly suggest that IL-21 regulates maturation of B cell progenitors and, in combination with anti-CD40, can lead to the formation of Ig-secreting cells.

Acknowledgments

We thank Rakesh Nayyar and Francis Tong for help with cell sorting.

Disclosures

The authors have no financial conflicts of interest.

References

1. Lu, L., P. Chaudhury, and D. G. Osmond. 1999. Regulation of cell survival during B lymphopoiesis: apoptosis and Bcl-2/Bax content of precursor B cells in bone marrow of mice with altered expression of IL-7 and recombinase-activating gene-2. *J. Immunol.* 162: 1931–1940.
2. Sudo, T., M. Ito, Y. Ogawa, M. Iizuka, H. Kodama, T. Kunisada, S. Hayashi, M. Ogawa, K. Sakai, and S. Nishikawa. 1989. Interleukin 7 production and function in stromal cell-dependent B cell development. *J. Exp. Med.* 170: 333–338.
3. Corcoran, A. E., F. M. Smart, R. J. Cowling, T. Crompton, M. J. Owen, and A. R. Venkitaraman. 1996. The interleukin-7 receptor α chain transmits distinct signals for proliferation and differentiation during B lymphopoiesis. *EMBO J.* 15: 1924–1932.
4. Miller, J. P., D. Izon, W. DeMuth, R. Gerstein, A. Bhandoola, and D. Allman. 2002. The earliest step in B lineage differentiation from common lymphoid progenitors is critically dependent upon interleukin 7. *J. Exp. Med.* 196: 705–711.
5. Fleming, H. E., and C. J. Paige. 2001. Pre-B cell receptor signaling mediates selective response to IL-7 at the pro-B to pre-B cell transition via an ERK/MAP kinase-dependent pathway. *Immunity* 15: 521–531.
6. Leonard, W. J., and R. Spolski. 2005. Interleukin-21: a modulator of lymphoid proliferation, apoptosis and differentiation. *Nat. Rev. Immunol.* 5: 688–698.
7. Ettinger, R., S. Kuchen, and P. E. Lipsky. 2008. The role of IL-21 in regulating B-cell function in health and disease. *Immunol. Rev.* 223: 60–86.

8. Konforte, D., N. Simard, and C. J. Paige. 2009. IL-21: an executor of B cell fate. *J. Immunol.* 182: 1781–1787.
9. Ozaki, K., R. Spolski, C. G. Feng, C. F. Qi, J. Cheng, A. Sher, H. C. Morse, III, C. Liu, P. L. Schwartzberg, and W. J. Leonard. 2002. A critical role for IL-21 in regulating immunoglobulin production. *Science* 298: 1630–1634.
10. Konforte, D., and C. J. Paige. 2006. Identification of cellular intermediates and molecular pathways induced by IL-21 in human B cells. *J. Immunol.* 177: 8381–8392.
11. de Totero, D., R. Meazza, S. Zupo, G. Cutrona, S. Matis, M. Colombo, E. Balleari, I. Pierri, M. Fabbi, M. Capaia, et al. 2006. Interleukin-21 receptor (IL-21R) is up-regulated by CD40 triggering and mediates proapoptotic signals in chronic lymphocytic leukemia B cells. *Blood* 107: 3708–3715.
12. Jin, H., R. Carrio, A. Yu, and T. R. Malek. 2004. Distinct activation signals determine whether IL-21 induces B cell costimulation, growth arrest, or Bim-dependent apoptosis. *J. Immunol.* 173: 657–665.
13. Ozaki, K., R. Spolski, R. Ettinger, H. P. Kim, G. Wang, C. F. Qi, P. Hwu, D. J. Shaffer, S. Akilesh, D. C. Roopenian, et al. 2004. Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. *J. Immunol.* 173: 5361–5371.
14. Jin, H., and T. R. Malek. 2006. Redundant and unique regulation of activated mouse B lymphocytes by IL-4 and IL-21. *J. Leukoc. Biol.* 80: 1416–1423.
15. Bubier, J. A., T. J. Sproule, O. Foreman, R. Spolski, D. J. Shaffer, H. C. Morse, III, W. J. Leonard, and D. C. Roopenian. 2009. A critical role for IL-21 receptor signaling in the pathogenesis of systemic lupus erythematosus in BXSB-Yaa mice. *Proc. Natl. Acad. Sci. USA* 106: 1518–1523.
16. Ozaki, K., A. Hishiya, K. Hatanaka, H. Nakajima, G. Wang, P. Hwu, T. Kitamura, K. Ozawa, W. J. Leonard, and T. Nosaka. 2006. Overexpression of interleukin 21 induces expansion of hematopoietic progenitor cells. *Int. J. Hematol.* 84: 224–230.
17. Pelletier, M., and D. Girard. 2006. Differential effects of IL-15 and IL-21 in myeloid ($CD11b^+$) and lymphoid ($CD11b^-$) bone marrow cells. *J. Immunol.* 177: 100–108.
18. Dienz, O., S. M. Eaton, J. P. Bond, W. Neveu, D. Moquin, R. Noubade, E. M. Briso, C. Charland, W. J. Leonard, G. Ciliberto, et al. 2009. The induction of antibody production by IL-6 is indirectly mediated by IL-21 produced by $CD4^+$ T cells. *J. Exp. Med.* 206: 69–78.
19. Milne, C. D., H. E. Fleming, and C. J. Paige. 2004. IL-7 does not prevent pro-B/pre-B cell maturation to the immature/$sIgM^+$ stage. *Eur. J. Immunol.* 34: 2647–2655.
20. Bryant, V. L., C. S. Ma, D. T. Avery, Y. Li, K. L. Good, L. M. Corcoran, R. de Waal Malefyt, and S. G. Tangye. 2007. Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by $CXCR5^+$ T follicular helper cells. *J. Immunol.* 179: 8180–8190.
21. Bikoff, E. K., M. A. Morgan, and E. J. Robertson. 2009. An expanding job description for Blimp-1/PRDM1. *Curr. Opin. Genet. Dev.* 19: 379–385.
22. Ettinger, R., G. P. Sims, A. M. Fairhurst, R. Robbins, Y. S. da Silva, R. Spolski, W. J. Leonard, and P. E. Lipsky. 2005. IL-21 induces differentiation of human naive and memory B cells into antibody-secreting plasma cells. *J. Immunol.* 175: 7867–7879.
23. Saito, T., D. Kitayama, A. Sakamoto, N. Tsuruoka, M. Arima, M. Hatano, M. Miyazaki, and T. Tokuhisa. 2008. Effective collaboration between IL-4 and IL-21 on B cell activation. *Immunobiology* 213: 545–555.
24. Pène, J., J. F. Gauchat, S. Lécart, E. Drouet, P. Guglielmi, V. Boulay, A. Delwail, D. Foster, J. C. Lecron, and H. Yssel. 2004. Cutting edge: IL-21 is a switch factor for the production of IgG1 and IgG3 by human B cells. *J. Immunol.* 172: 5154–5157.
25. Avery, D. T., V. L. Bryant, C. S. Ma, R. de Waal Malefyt, and S. G. Tangye. 2008. IL-21–induced isotype switching to IgG and IgA by human naive B cells is differentially regulated by IL-4. *J. Immunol.* 181: 1767–1779.
26. Nurieva, R., X. O. Yang, G. Martinez, Y. Zhang, A. D. Panopoulos, L. Ma, K. Schluns, Q. Tian, S. S. Watowich, A. M. Jetten, and C. Dong. 2007. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* 448: 480–483.
27. Coquet, J. M., K. Kyparissoudis, D. G. Pellicci, G. Besra, S. P. Berzins, M. J. Smyth, and D. I. Godfrey. 2007. IL-21 is produced by NKT cells and modulates NKT cell activation and cytokine production. *J. Immunol.* 178: 2827–2834.
28. Harada, M., K. Magara-Koyanagi, H. Watarai, Y. Nagata, Y. Ishii, S. Kojo, S. Horiguchi, Y. Okamoto, T. Nakayama, N. Suzuki, et al. 2006. IL-21–induced Bε cell apoptosis mediated by natural killer T cells suppresses IgE responses. *J. Exp. Med.* 203: 2929–2937.
29. Chtanova, T., S. G. Tangye, R. Newton, N. Frank, M. R. Hodge, M. S. Rolph, and C. R. Mackay. 2004. T follicular helper cells express a distinctive transcriptional profile, reflecting their role as non-Th1/Th2 effector cells that provide help for B cells. *J. Immunol.* 173: 68–78.
30. Cariappa, A., C. Chase, H. Liu, P. Russell, and S. Pillai. 2007. Naive recirculating B cells mature simultaneously in the spleen and bone marrow. *Blood* 109: 2339–2345.
31. Lindsley, R. C., M. Thomas, B. Srivastava, and D. Allman. 2007. Generation of peripheral B cells occurs via two spatially and temporally distinct pathways. *Blood* 109: 2521–2528.
32. Pillai, S., and A. Cariappa. 2009. The bone marrow perisinusoidal niche for recirculating B cells and the positive selection of bone marrow-derived B lymphocytes. *Immunol. Cell Biol.* 87: 16–19.
33. Parrish-Novak, J., S. R. Dillon, A. Nelson, A. Hammond, C. Sprecher, J. A. Gross, J. Johnston, K. Madden, W. Xu, J. West, et al. 2000. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. *Nature* 408: 57–63.
34. Han, J. H., S. Akira, K. Calame, B. Beutler, E. Selsing, and T. Imanishi-Kari. 2007. Class switch recombination and somatic hypermutation in early mouse B cells are mediated by B cell and Toll-like receptors. *Immunity* 27: 64–75.
35. Ueda, Y., D. Liao, K. Yang, A. Patel, and G. Kelsoe. 2007. T-independent activation-induced cytidine deaminase expression, class-switch recombination, and antibody production by immature/transitional 1 B cells. *J. Immunol.* 178: 3593–3601.
36. Kuraoka, M., D. Liao, K. Yang, S. D. Allgood, M. C. Levesque, G. Kelsoe, and Y. Ueda. 2009. Activation-induced cytidine deaminase expression and activity in the absence of germinal centers: insights into hyper-IgM syndrome. *J. Immunol.* 183: 3237–3248.
37. Sugiyama, H., T. Maeda, S. Akira, and S. Kishimoto. 1986. Class-switching from μ to γ3 or γ2b production at pre-B cell stage. *J. Immunol.* 136: 3092–3097.
38. DePinho, R., K. Kruger, N. Andrews, S. Lutzker, D. Baltimore, and F. W. Alt. 1984. Molecular basis of heavy-chain class switching and switch region deletion in an Abelson virus-transformed cell line. *Mol. Cell. Biol.* 4: 2905–2910.
39. Burrows, P. D., G. B. Beck, and M. R. Wabl. 1981. Expression of μ and γ immunoglobulin heavy chains in different cells of a cloned mouse lymphoid line. *Proc. Natl. Acad. Sci. USA* 78: 564–568.
40. Kallies, A., J. Hasbold, D. M. Tarlinton, W. Dietrich, L. M. Corcoran, P. D. Hodgkin, and S. L. Nutt. 2004. Plasma cell ontogeny defined by quantitative changes in blimp-1 expression. *J. Exp. Med.* 200: 967–977.
41. Seagal, J., E. Edry, Z. Keren, N. Leider, O. Benny, M. Machluf, and D. Melamed. 2003. A fail-safe mechanism for negative selection of isotype-switched B cell precursors is regulated by the Fas/FasL pathway. *J. Exp. Med.* 198: 1609–1619.
42. Orinska, Z., A. Osiak, J. Löhler, E. Bulanova, V. Budagian, I. Horak, and S. Bulfone-Paus. 2002. Novel B cell population producing functional IgG in the absence of membrane IgM expression. *Eur. J. Immunol.* 32: 3472–3480.
43. Macpherson, A. J., A. Lamarre, K. McCoy, G. R. Harriman, B. Odermatt, G. Dougan, H. Hengartner, and R. M. Zinkernagel. 2001. IgA production without μ or δ chain expression in developing B cells. *Nat. Immunol.* 2: 625–631.
44. Di Rosa, F. 2009. T-lymphocyte interaction with stromal, bone and hematopoietic cells in the bone marrow. *Immunol. Cell Biol.* 87: 20–29.
45. Price, P. W., and J. Cerny. 1999. Characterization of $CD4^+$ T cells in mouse bone marrow. I. Increased activated/memory phenotype and altered TCR Vβ repertoire. *Eur. J. Immunol.* 29: 1051–1056.
46. Monteiro, J. P., A. Benjamin, E. S. Costa, M. A. Barcinski, and A. Bonomo. 2005. Normal hematopoiesis is maintained by activated bone marrow $CD4^+$ T cells. *Blood* 105: 1484–1491.
47. Cassese, G., E. Parretta, L. Pisapia, A. Santoni, J. Guardiola, and F. Di Rosa. 2007. Bone marrow CD8 cells down-modulate membrane IL-7Rα expression and exhibit increased STAT-5 and p38 MAPK phosphorylation in the organ environment. *Blood* 110: 1960–1969.
48. Pulle, G., M. Vidric, and T. H. Watts. 2006. IL-15–dependent induction of 4-1BB promotes antigen-independent CD8 memory T cell survival. *J. Immunol.* 176: 2739–2748.
49. Sabbagh, L., L. M. Snell, and T. H. Watts. 2007. TNF family ligands define niches for T cell memory. *Trends Immunol.* 28: 333–339.
50. Li, Y., G. Toraldo, A. Li, X. Yang, H. Zhang, W. P. Qian, and M. N. Weitzmann. 2007. B cells and T cells are critical for the preservation of bone homeostasis and attainment of peak bone mass in vivo. *Blood* 109: 3839–3848.
51. Ueda, Y., K. Yang, S. J. Foster, M. Kondo, and G. Kelsoe. 2004. Inflammation controls B lymphopoiesis by regulating chemokine CXCL12 expression. *J. Exp. Med.* 199: 47–58.
52. Cain, D., M. Kondo, H. Chen, and G. Kelsoe. 2009. Effects of acute and chronic inflammation on B-cell development and differentiation. *J. Invest. Dermatol.* 129: 266–277.