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Regulation of B Cell Differentiation and Plasma Cell Generation by IL-21, a Novel Inducer of Blimp-1 and Bcl-6[1]

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IL-21 is a type I cytokine whose receptor is expressed on T, B, and NK cells. Within the B cell lineage, IL-21 regulates IgG1 production and cooperates with IL-4 for the production of multiple Ab classes in vivo. Using IL-21-transgenic mice and hydrodynamics-based gene delivery of IL-21 plasmid DNA into wild-type mice as well as in vitro studies, we demonstrate that although IL-21 induces death of resting B cells, it promotes differentiation of B cells into postswitch and plasma cells. Thus, IL-21 differentially influences B cell fate depending on the signaling context, explaining how IL-21 can be proapoptotic for B cells in vitro yet critical for Ag-specific Ig production in vivo. Moreover, we demonstrate that IL-21 unexpectedly induces expression of both Blimp-1 and Bcl-6, indicating mechanisms as to how IL-21 can serve as a complex regulator of B cell maturation and terminal differentiation. Finally, BXSB-*Yaa* mice, which develop a systemic lupus erythematosus-like disease, have greatly elevated IL-21, suggesting a role for IL-21 in the development of autoimmune disease. *The Journal of Immunology,* **2004, 173: 5361–5371.**

Interleukin 21 is a type I cytokine whose receptor is most closely related to the IL-2R β-chain (1–3). Like IL-2, IL-4, IL-7, IL-9, and IL-15, IL-21 utilizes the common cytokine receptor γ-chain (γ_c)[6] (4) and signals in part through the activation of Jak1 and Jak3 as well as Stat1, Stat3, and Stat5 (1, 4). IL-21 is produced by activated $CD4^+$ T cells and the IL-21R is expressed on T, B, and NK cells (1, 2). In vitro, IL-21 can act as a comitogen for anti-CD3-induced thymocyte and peripheral T cell proliferation (2), augment NK cell expansion and differentiation from human $CD34^+$ cells when cultured with IL-15 and Flt-3 ligand (2), and can also activate NK cytolytic activity (2, 5). We previously demonstrated that IL-21 critically regulates Ig production (6). IL-$21R^{-/-}$ mice have markedly diminished IgG1 but greatly elevated IgE levels in response to Ag and, correspondingly, IL-21 can inhibit Ag-induced IgE production (7). Mice lacking expression of both IL-21R and IL-4 exhibit a dysgammaglobulinemia with severely impaired IgG and IgE responses (6). In vitro, IL-21 can enhance the proliferative response of human and murine B cells stimulated with Abs to CD40, but it inhibits B cell proliferation in response to anti-IgM plus IL-4 (2) and correspondingly can augment B cell death (8). Using IL-21-transgenic (TG) mice and hydrodynamic injection of IL-21 plasmid-based methodologies, we demonstrate that IL-21 induces apoptosis in a subset of mature B cells but increases the number of immature and postswitch B cells. Moreover, IL-21 induces plasma cell differentiation. We show that IL-21 is an inducer of both Blimp-1 and Bcl-6 and, interestingly, a down-regulator of CD23. Finally, we show greatly elevated levels of IL-21 in the BXSB-*Yaa* mouse model of systemic lupus erythematosus (SLE). Thus, we establish new roles for IL-21 in B cell biology, with important implications for the basis of the development of autoimmunity.

Materials and Methods

Splenic B cell preparation and proliferation assays

C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Splenic B cells were isolated using B220 or CD43 magnetic beads (Miltenyi Biotec, Auburn, CA) and were >95% pure as assessed by flow cytometry. B cells were plated in 96-well plates at 10^5 cells/well and treated as indicated with anti-CD40 (1 μg/ml; BD Pharmingen, San Diego, CA), anti-IgM (5 μg; Sigma-Aldrich, St. Louis, MO), murine IL-4 (200 U/ml), and murine IL-21 (50 ng/ml). For proliferation assays, cells were cultured for 2 days and pulsed with [^{3}H]thymidine (1 μCi/well) for the last 18 h of culture.

Western blotting

Clarified whole cell lysates were subjected to SDS-PAGE and Western blotting with rabbit anti-Blimp1 (kind gift from Dr. M. Davis). Blots were developed with an ECL substrate (Amersham Biosciences, Piscataway, NJ).

Staining for apoptotic cells

Apoptosis was assessed using annexin V and 7-aminoactinomycin D (BD Pharmingen) and the TUNEL staining reagent (Roche Applied Science, Indianapolis, IN).

TG mice

Murine or human IL-21 cDNA constructs containing V5 and His tags were generated by PCR and inserted into pHSE, a plasmid in which the expressed cDNA is under the control of the H-$2k^b$ promoter and IgM enhancer (9, 10). TG mice were then generated by standard procedures.

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[6] Abbreviations used in this paper: γ_c, common γ-chain; TG, transgenic; MAdCAM-1, mucosal addressin cell adhesion molecule 1; FW, forward; RV, reverse; TP, TaqMan probe; GPR, global pattern recognition; MZ, marginal zone; WT, wild type; SLE, systemic lupus erythematosus; KO, knockout.

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In vivo transient expression of IL-21

The murine IL-21 cDNA was subcloned into the pORF expression vector (InvivoGen, San Diego, CA), and 20 μg of DNA in 2 ml of saline was injected i.v. into C57BL/6 mice within 5 s (hydrodynamics-based transfection) (11, 12). IL-21 delivered in this fashion results in a serum level of ~6 ng/ml at day 1 that disappeared by day 8 (12). Mice injected with IL-21 or control mice injected with saline or pORF were analyzed at day 7.

Flow cytometric analysis of B cell populations

Cell populations were stained with the following Abs: FITC anti-CD21, CD23, IgG and IgM, PE anti-CD23 and IgD, allophycocyanin anti-B220 (BD Pharmingen). Staining with CD19, Syndecan-1, CD1d, AA4.1, IgG1(A85-1), and CD9 Abs was revealed with either PE- or Cy-conjugated streptavidin (BD Pharmingen). Bcl-2 staining was preceded by fixation with Cytofix/Cytoperm (BD Pharmingen). Data were collected using a FACSCalibur flow cytometer and analyzed using CellQuest software (BD Immunosystems, San Jose, CA). An Ab to CD16 (24G2;,BD Pharmingen) was used to block FcR binding. In addition, splenocytes were preincubated at 37°C for 2 h before staining to further minimize the possibility of cytophilic Ig retention on the cell surface.

Immunohistochemical staining of lymphoid follicles

Spleens were analyzed by either immunohistology or flow cytometry. Tissues were embedded in Tissue-Tek/OCT compound (Sakura, Zoeterwoude, The Netherlands), frozen in liquid nitrogen, serially sectioned, immediately fixed in ice-cold acetone for 5 min, and stained for 45 min in a humid chamber with either biotinylated mucosal addressin cell adhesion molecule 1 (MAdCAM-1; Southern Biotechnology Associates, Birmingham, AL), rat Ab supernatant specific for IgD (clone 1126C), or purified rat Ab specific for MCA1849 (MARCO; Serotec, Raleigh, NC). The sections were washed and bound Abs were revealed with either streptavidin-conjugated or goat anti-rat conjugated Oregon Green (Molecular Probes, Eugene, OR). IgM was detected with goat anti-mouse IgM-Texas Red (Southern Biotechnology Associates).

EMSAs

Nuclear extracts were prepared from splenic B cells that were cultured with anti-IgM with or without IL-21 for 24 h. Five micrograms was used for DNA binding reactions with either a Blimp-1 binding site (MHC2TA) from the class II MHC promoter (13) or with a Bcl-6 consensus binding site (14).

Real-time PCR

Blimp-1, Bcl-6, and Pax5 mRNA levels were quantitated relative to GAPDH mRNA levels by real-time PCR. RNA was reverse transcribed using an Omniscript kit (Qiagen, Valencia, CA) according to the manufacturer's directions and PCR was performed using a Quantitect Probe Detection system (Qiagen) and the following oligonucleotides: Blimp-1 forward (FW), 5′-ACAGAGGCCGAGTTTGAAGAGA-3′; reverse (RV), 5′-AAGGATGCCTCGGCTTGAA-3′; TaqMan probe (TP), 5′-[6-FAM] CCCTGGGATTCCGGCGCTG[TAMRA-6-FAM]-3′; Pax5 FW, 5′-AAACGCAAGAGGGATGAAGGT-3′; RV, 5′-AACAGGTCTCCCCG CATCT-3′; TP, 5′-[6-FAM]CACTTCCGGGCCGGGACTTCC[TAMRA 6-FAM]-3′; BCL-6 FW, 5′-TCAGAGTATTCGGATTCTAGCTGTGA-3′; RV, 5′-TGCAGCGTGTGCCTCTTG-3′; TP, 5′-[6-FAM]TGCAACGA ATGTGACTGCCGTTTCTCT[TAMRA-6-FAM]-3′; GAPDH FW, 5′-TTCACCACCATGGAGAAGGC-3′; RV, 5′-GGCATGGACTGTGGT CATGA-3′; and TP, 5′-[6-FAM] TGCATCCTGCACCACCAACTGCT TAG[TAMRA-6-FAM]-3′.

*Analysis of gene expression in BXSB-*Yaa *mouse spleens by quantitative ImmunoQuantArray real-time PCR*

Total RNA was prepared from BXSB/MpJ-*Yaa* and BXSB.B6-*Yaa*$^+$/Dcr spleens and cDNA synthesis from 5–10 μg of total RNA was conducted using the Retroscript kit (Ambion, Austin, TX). This ImmunoQuantArray consisted of 384 oligonucleotide primer pairs (15) designed to specifically amplify a customized set of target gene cDNAs. Primers for the genes discussed in the text are: IgG forward, 5′-CACCTCCCAAGGAGCAGA TG-3′; reverse, 5′-CCCAGTTGCTCTTCTGCACAT-3′; IgG2b forward, 5′-CATCACCCATCGAGAGAACCA-3′; reverse, 5′-ACACTGATGT CTCCAGGGTTGA-3′; IgG3 forward, 5′-CAGCCAGCAAGACTGAGT TGAT-3′; reverse, 5′-GTCATCCTCGCTCACATCCA-3′; IL-21 forward, 5′-CCTGGAGTGGTATCATCGCTTT-3′; and reverse, 5′-TGATTGTG ACACTTTTCTGGGAAT-3′.

Quantatitive expression analysis was performed essentially as described (15) using SYBR Green detection (Applied Biosystems, Foster City, CA). The global pattern recognition (GPR) algorithm (15) was used to identify significant changes in gene expression. A detailed description of the GPR software is available at http://www.jax.org/staff/roopenian/labsite/gene_expression.html). In this study, 208 of the 384 genes examined qualified as normalizers; fold changes are based on one of these, 18S RNA.

Analysis of serum IL-21, IgG1, and IgG3 levels

Serum levels of murine IL-21 were measured by sandwich ELISA using a rat capture mAb (149215.111) and biotinylated goat Ab (BAF594) specific for murine IL-21 (R&D Systems, Minneapolis, MN). IgG1 and IgG3 levels were measured using capture/detection Abs from BD Pharmingen.

Results

IL-21 increases immature B cell numbers and drives B cells to differentiate into postswitch cells

The proapoptotic effects of IL-21 on B cells in vitro (8) were surprising given that IL-21R expression is essential for normal Ig production in vivo (6). To help explain this apparent paradox, we generated IL-21 TG mice using a vector (9, 10) that drives expression in T, B, and NK cells. For unclear reasons, founder mice expressing murine IL-21 uniformly exhibited growth retardation and died before sexual maturity. We therefore generated TG mice expressing human IL-21, which can stimulate murine cells in vitro but which we hypothesized might bind to the murine IL-21R with lower affinity than murine IL-21. Four founders of the human IL-21 TG mice exhibited growth retardation and died before adulthood, but three viable lines were obtained (Fig. 1*A*). The line with the greatest IL-21 mRNA expression was lost, consistent with toxicity resulting from high expression of IL-21; therefore, we focused on the two lower expressing lines (#5 and #7).

In normal mouse spleen, ~90% of B cells are mature follicular or marginal zone (MZ) cells (16). Immature or newly formed B cells are produced in the bone marrow and migrate to the spleen as transitional T1 cells where they mature into transitional T2 cells. Only 1–3% of these cells differentiate into mature B cells (17, 18), which can develop into memory B cells and Ab-forming plasma cells after Ag stimulation (19, 20). Staining of splenocytes from the human IL-21 TG and wild-type (WT) mice with Abs to IgM, CD21, and CD23 suggested that mature B cells and transitional T2/MZ B cell populations were decreased, whereas transitional T1 cells appeared to be intact or increased in the IL-21 TG mice (Fig. 2*A*, *ii* vs *i*). However, we found that IL-21 potently decreases CD23 expression on mature B cells (Fig. 1*B*) and modestly diminishes expression of CD21 (data not shown), making CD21 and CD23 unreliable as phenotypic markers of B cell subsets, similar to what has been reported in BAFF-deficient mice (21).

We therefore stained cells with AA4.1 mAb, which binds to a protein expressed on immature B cells (22), as well as with IgM and IgD which are not affected by IL-21 (data not shown), to characterize B cell populations. Consistent with an increase in immature B cells, AA4.1^{high} cells were increased in both IL-21 TG (Fig. 1*C*, *ii* vs *i*) and IL-21-injected mice (Fig. 2*C*, *ii* vs *i*), although the IgM/IgD staining pattern revealed no significant differences in the T1:T2 B cell ratio (Figs. 1*D* and 2*D*). The percentage of IgD^{low}IgM^{high}AA4.1^{low} MZ cells in either the IL-21 TG or IL-21-injected mice (Figs. 1 and 2*D*, *iv* vs *iii*, *lower right quadrants*) were similar to those found in control WT mice. B1 B cell numbers were also similar in both the spleens and peritoneal cavities of WT and IL-21 TG mice based on CD5 and CD43 staining (data not shown). The AA4.1^{low} mature splenic B cell population exhibited a decrease in the percentage of the most mature follicular B cell subset (IgD^{high}IgM^{low}; Fig. 1*D*, *iv* vs *iii*, *upper left quadrant*). However, the absolute number of mature cells (B220$^+$AA4.1^{low})

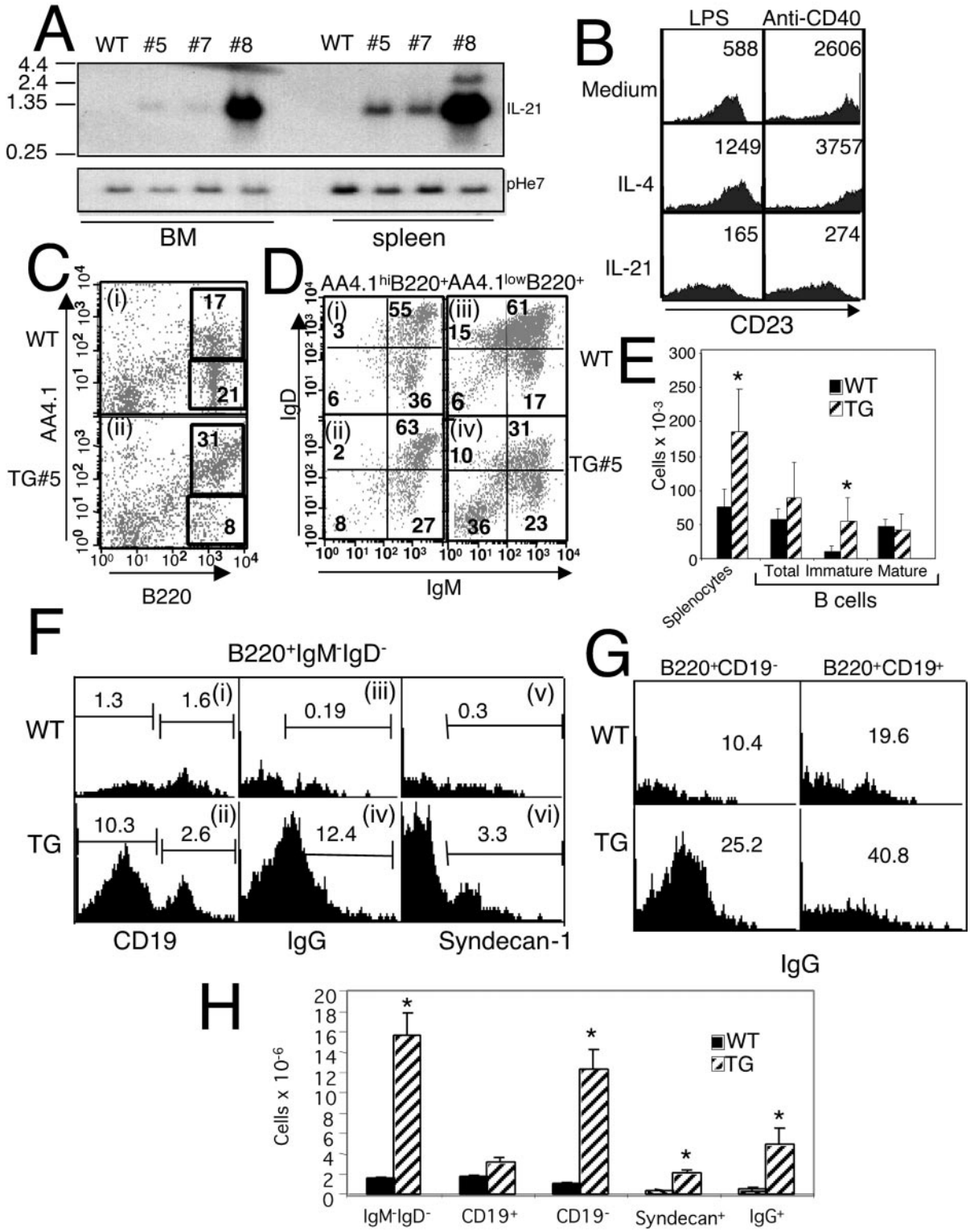

FIGURE 1. IL-21 increases immature and postswitch B cells. *A,* Expression of IL-21 mRNA in IL-21 TG mice. Total RNA (10 μg) from bone marrow or spleen from WT or IL-21 TG mice was Northern blotted with human IL-21 cDNA (*upper panel*) or control pHe7 (*lower panel*) probes. The levels of IL-21 protein being produced in these animals could not be determined, as in contrast to our ability to perform ELISAs for murine IL-21 (12), we do not have mAbs for human IL-21 appropriate for ELISAs. *B,* CD23 expression is decreased by IL-21 but increased by IL-4. Purified B cells treated with LPS or anti-CD40 were additionally stimulated with medium, IL-4, or IL-21 for 10 h and then CD23 expression on viable cells was determined by flow cytometry. Shown are mean fluorescent intensities. *C,* Flow cytometric analysis of B cell populations. AA4.1/B220 staining of splenocytes from WT or TG mice (*i* and *ii*) is shown. *D,* IgM/IgD profiles are shown for AA4.1^{high}B220^{+} (*i* and *ii*) and AA4.1^{low}B220^{+} (*iii* and *iv*) splenocytes. *E,* Cellularity in WT vs IL-21 TG mice. Shown are total cell number $\pm$ SD (from >10 animals) for splenocytes and total B cells (defined by B220) as well as AA4.1^{high} immature and AA4.1^{low} mature B cell subpopulations. *F,* B220^{+}IgM^{-}IgD^{-} splenocytes from WT and IL-21 TG mice were analyzed for CD19, surface IgG, and Syndecan-1 staining. Shown is the absolute number of cells per mouse $\times$ 10^{-6}. *G,* B220^{+}CD19^{-} and B220^{+}CD19^{+} splenocytes from WT and IL-21 TG mice were analyzed for surface IgG. *H,* Summary of numbers of IgM^{-}IgD^{-}, CD19^{+}, CD19^{-}, Syndecan-1^{+}, and IgG^{+} cells from WT and IL-21 TG mice. $*$, $p < 0.01$ for TG mice compared with WT in *E* and *H*.

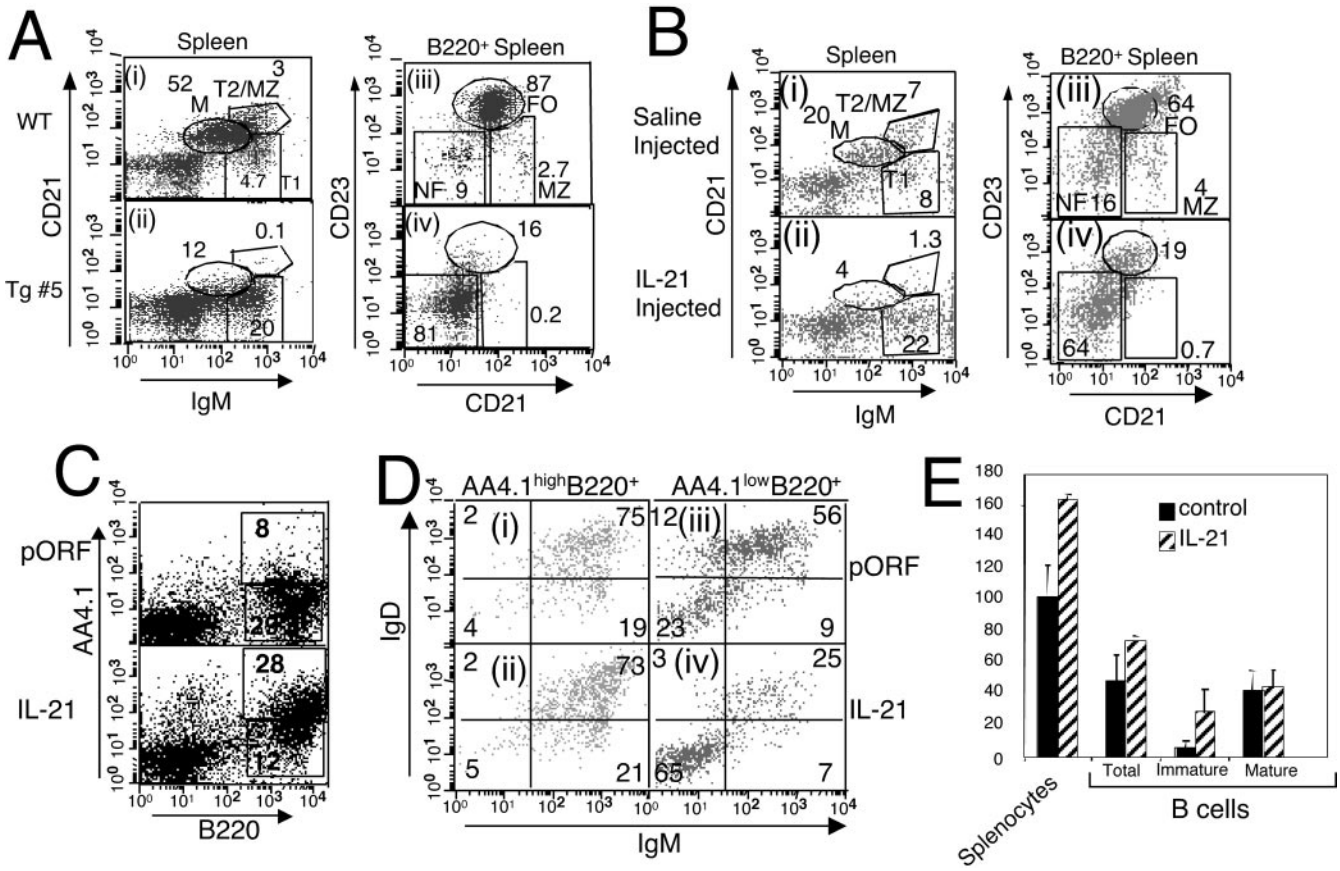

FIGURE 2. Analysis of IL-21 TG or WT mice injected with a murine IL-21 plasmid by hydrodynamic transfection. *A* and *B,* Lower apparent numbers of mature (M), follicular (FO), and MZ B cells in IL-21 TG mice (*A*) or IL-21-injected mice (*B*) than in control mice based on CD21/CD23 and IgM/CD21 splenic expression patterns. T1, transitional B cell 1; T2, transitional B cell 2; NF, newly formed B cells. Injected mice were analyzed at day 7. *C,* Increased AA4.1$^+$ B cells in IL-21 plasmid-injected as compared with pORF-injected mice. Staining was with AA4.1 vs B220. *D,* Shown are IgD vs IgM profiles of either AA4.1^{high}/B220$^+$ (*i* and *ii*) or AA4.1^{low}/B220$^+$ (*iii* and *iv*) splenocytes. *i* and *iii,* Mice were injected with pORF, whereas in *ii* and *iv*, mice were injected with the murine IL-21 plasmid. *E,* Cellularity in control vs IL-21 plasmid-injected mice. Shown are total cell numbers ± SD (from >10 animals) for splenocytes and total B cells (defined by B220) as well as AA4.1^{high} immature and AA4.1^{low} mature B cell subpopulations.

was not altered in either the IL-21 TG (Fig. 1*E*) or the IL-21-injected mice (Fig. 2*E*). Interestingly, in both IL-21 TG mice and IL-21-injected mice (Figs. 1*D* and 2*D*, *iv* vs *iii*, *left lower quadrants*), there was a dramatic increase in the IgD$^-$IgM$^-$ population of AA4.1^{low} splenocytes. This population was further characterized and was found to contain a significantly increased percentage of B220$^+$CD19$^-$ cells (Fig. 1*F*, *ii* vs *i*). In addition, the percentage and absolute number of cells in this population which express either IgG or Syndecan-1 were markedly increased in IL-21 TG mice (Fig. 1*F*, *iv* and *vi* vs *ii* and *v*; the numbers indicate the absolute number of cells × 10^{-6}/mouse), with IgG expression being increased on both B220$^+$CD19$^-$ and B220$^+$CD19$^+$ cells (Fig. 1*G*). Overall, based on the AA4.1 and IgM/IgD staining, IL-21 increased the total number of splenocytes and total B cells, with a marked increase in the number of immature AA4.1^{high} B cells (summarized in Figs. 1*E* and 2*E* for IL-21 TG and IL-21 plasmid-injected mice, respectively). The total number of mature B cells (including postswitch cells; together defined as AA4.1^{low} cells), was normal (Figs. 1*E* and 2*E*). However, of these cells, the percentage of mature B cells was slightly diminished based on the decrease in IgD^{high} cells in Figs. 1*D* and 2*D* (*iv* vs *iii*, *upper left quadrants*), whereas IgD$^-$IgM$^-$ postswitch B cells were increased (Figs. 1*D* and 2*D*, *iv* vs *iii*, *lower left quadrants*). Thus, IL-21 stimulation increases the number of immature B cells in the periphery and drives B cell differentiation into postswitch cells. The total numbers of IgM$^-$IgD$^-$, CD19$^+$, CD19$^-$, Syndecan-1$^+$, and IgG$^+$ cells in WT and IL-21 TG mice are summarized in Fig. 1*H*.

Maintenance of basic follicular structure in the spleen

Because of the dramatic effect of IL-21 on the differentiation of B cell subpopulations, we examined the effect of IL-21 on the architecture of the splenic white pulp. Immunostaining with Abs to IgM, IgD, MAdCAM-1 (to define marginal sinus), and MARCO (to define MZ macrophages) showed that spleens from IL-21 TG mice had intact follicular and MZ structures (Fig. 3, *D–F* vs *A–C*). Follicles were also readily identified in spleens from IL-21-injected mice, although there was a loss of MZ B cells as revealed by the lack of a bright red ring of IgM^{high}IgD^{low} B cells around the IgM^{low}IgD^{high}"green" follicles (Fig. 3, *G* vs *A*). Nevertheless, the marginal sinus and MZ macrophages, as defined by MAdCAM-1 (Fig. 3, *H* vs *B*) and MARCO (Fig. 3, *I* vs *C*) Abs, respectively, were still present in these mice, indicating a loss of B cells from this region rather than a loss of the MZ structure. The distributions of follicular dendritic cells as well as CD4$^+$ and CD8$^+$ T cells were normal in both IL-21-injected and TG mice (data not shown). Thus, chronic human IL-21 signaling in the TG mice did not affect the overall MZ structure, but led to an increase in the number of immature B cells and accumulation of Ig class-switched B cells. The IL-21-injected mice had similar changes in splenic B cell populations, except that the MZ B cells were not detected in the MZ

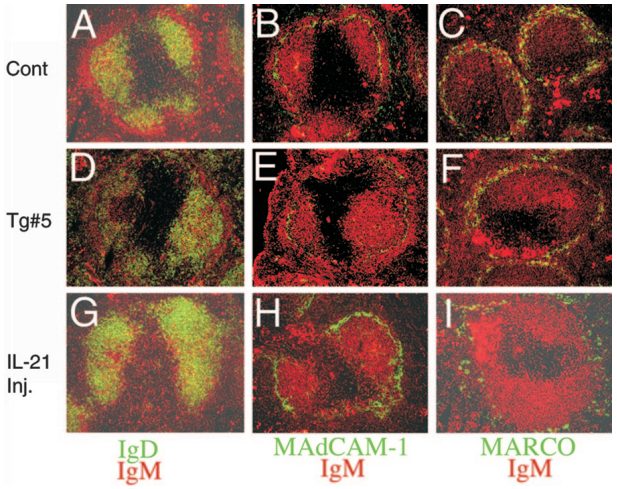

FIGURE 3. Basic splenic follicular structure is maintained following exposure to IL-21. Immunohistochemistry was performed on WT (control; *A–C*), TG (Tg #5; *D–F*), and IL-21-injected mice (*G–I*) using Abs to IgD/IgM (*A*, *D*, and *G*), MAdCAM-1/IgM (*B*, *E*, and *H*), and MARCO/IgM (*C*, *F*, and *I*). Data are representative of several mice examined.

(see loss of the "red" ring of cells in Fig. 3*G* vs its presence in 3*A*). We believe that these cells in fact are still present, based on CD1d and CD9 immunostaining (data not shown), but presumably have just migrated out of the MZ. This idea is supported by the retention of $IgM^{high}IgD^{low}AA4.1^{low}$ cells as evaluated by flow cytometry (Fig. 2*D*), which likely represents this MZ population of cells. The reason for the apparent redistribution of MZ cells is unclear, but importantly, both IL-21 TG and mice injected with IL-21 plasmid otherwise gave similar results.

Corresponding to the ability of IL-21 to induce apoptosis, higher annexin V staining of $B220^+$ B cells was seen in IL-21 TG mice than in WT littermates (Fig. 4*Ai*) and, similarly, injection of mice with the IL-21 plasmid increased annexin V staining (Fig. 4*Aii*). Although FACS and cell number analysis showed that there is not an overall decrease in the number of mature B cells in the IL-21 TG mice, these annexin V staining experiments suggest that IL-21 can initiate early steps in the apoptotic process in vivo but that these do not necessarily progress to cell death. Nevertheless, as shown above in Figs. 1 and 2, IL-21 also induced an increase in Ig class-switched B cells. Correspondingly, IL-21 TG mice had increased levels of serum IgG1 and IgM (Fig. 4*B*), and increased numbers of surface $IgG1^+$ expressing splenic B cells (Fig. 4*C*). Furthermore, IL-21-injected mice exhibited increased numbers of

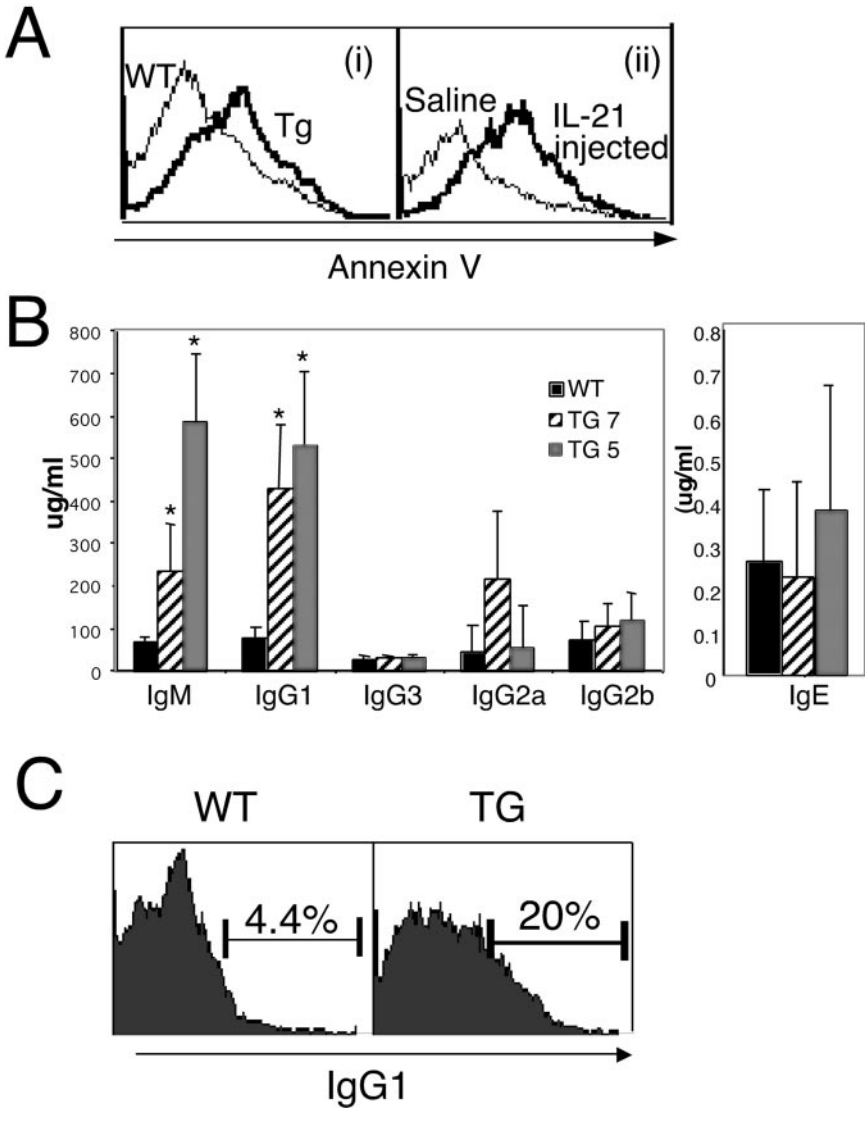

FIGURE 4. IL-21 decreases viability of splenic B cells but increases Ig class-switched B cells, with increased serum IgG1 and IgM and surface IgG1 expression on splenic B cells. *A,* Annexin V staining of $B220^+$ splenic B cells from TG line #5 mice (*i*) or IL-21 vector-injected mice (*ii*). *B,* Serum isotype levels (mean ± SD) in WT and two IL-21 TG lines; five mice were analyzed in each group. *, $p < 0.01$ for serum IgM and IgG1 in the TG mice as compared with WT. *C,* Increased surface $IgG1^+$ B cells in IL-21 TG mice. Splenocytes from IL-21 TG mice (line #5) and WT littermates were stained with anti-IgG1 and analyzed by flow cytometry.

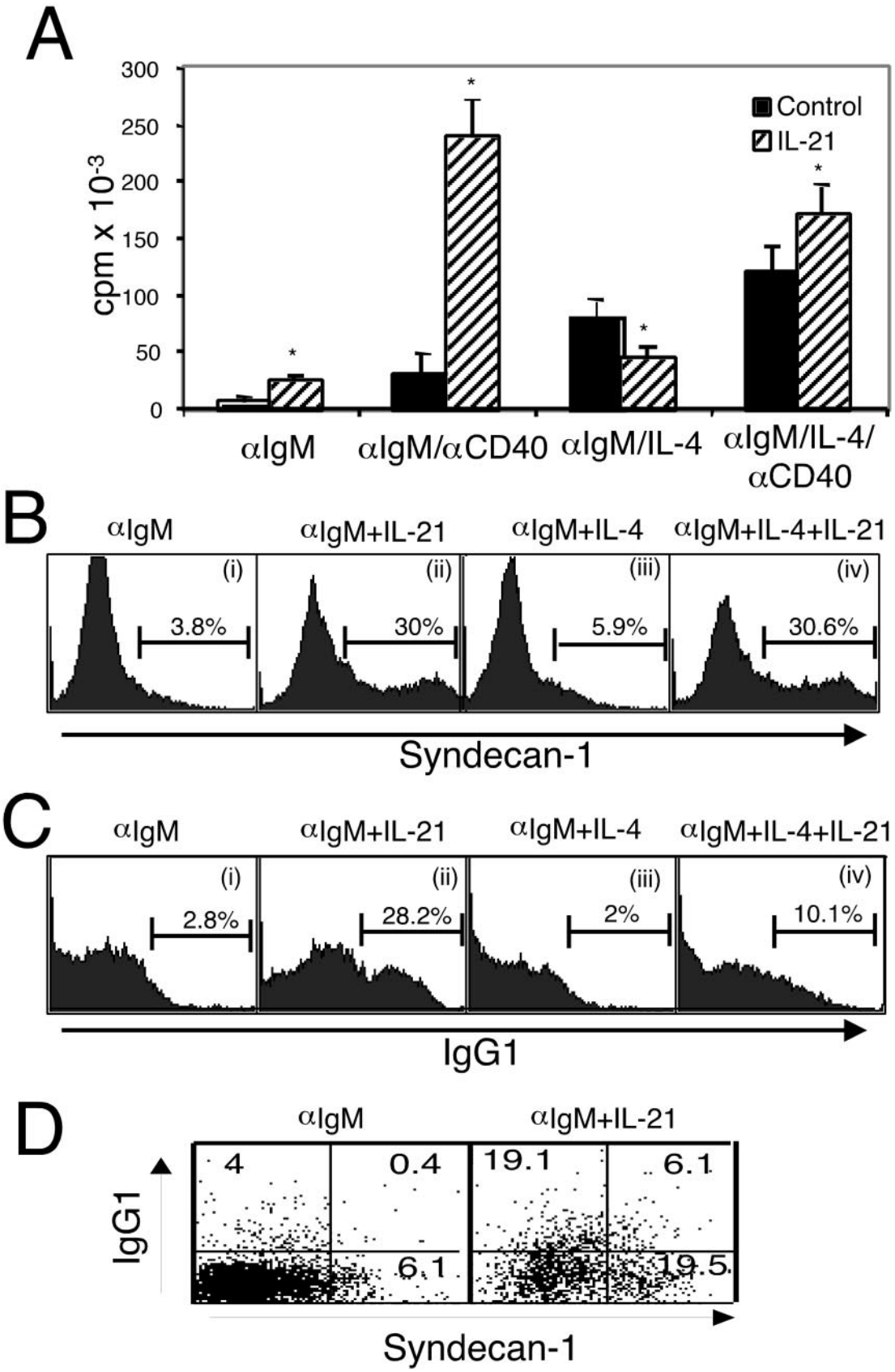

FIGURE 5. Direct effects of IL-21 on anti-IgM and/or anti-CD40-induced B cell proliferation and differentiation. *A,* IL-21 potently increased proliferation of B cells stimulated with anti-IgM + anti-CD40. Purified B cells from C57BL/6 WT mice were cultured with anti-IgM in the presence or absence of IL-21, IL-4, and anti-CD40 for 48 h and were then pulsed with [^{3}H]thymidine for the last 10 h. Shown is the average proliferative response of three mice analyzed in a representative experiment. *, $p < 0.01$ for IL-21 stimulated proliferation compared with control. *B–D,* Flow cytometric analysis of B cells cultured for 48 h as described above and analyzed for expression of Syndecan-1 (*B*) and surface IgG1 (*C*) or both (*D*). Data are representative of three similar experiments.

splenic B cells expressing cytoplasmic IgG, as evaluated by immunohistology (data not shown). The increased production of IgM as well as IgG1 is consistent with IL-21-induced differentiation of B cells into Ig-secreting cells, independent of isotype switching.

IL-21 can act directly on purified B cells

The effects of IL-21 on B cells could either be direct or indirect. We analyzed the effect of IL-21 on purified splenic B cells cultured for 48 h in the presence of anti-IgM in combination with anti-CD40, IL-4, or both stimuli (Fig. 5*A*). IL-21 increased B cell proliferation induced by anti-IgM, especially in the presence of anti-CD40 (Fig. 5*A*). Although IL-21 inhibited proliferation induced by anti-IgM plus IL-4, proliferation increased when anti-CD40 was added as a further stimulus, and the combination of anti-IgM plus IL-4 plus anti-CD40 gave slightly more proliferation in the presence than in the absence of IL-21 (Fig. 5*A*). Thus, in the presence of certain signals, such as the combination of B cell receptor stimulation and anti-CD40, IL-21 induced the greatest proliferation, but in the absence of either B cell receptor or anti-CD40 stimulation, IL-21 induced much less proliferation (Fig. 5). Consistent with the effect of TG IL-21 on increasing Ab production (Fig. 4*B*), IL-21 induced expression of Syndecan-1 (CD138, a plasma cell marker) and surface IgG1 (Fig. 5, *B* and *C*, respectively; see *ii* vs *i* and *iv* vs *iii*) in B cells stimulated with anti-IgM with or without

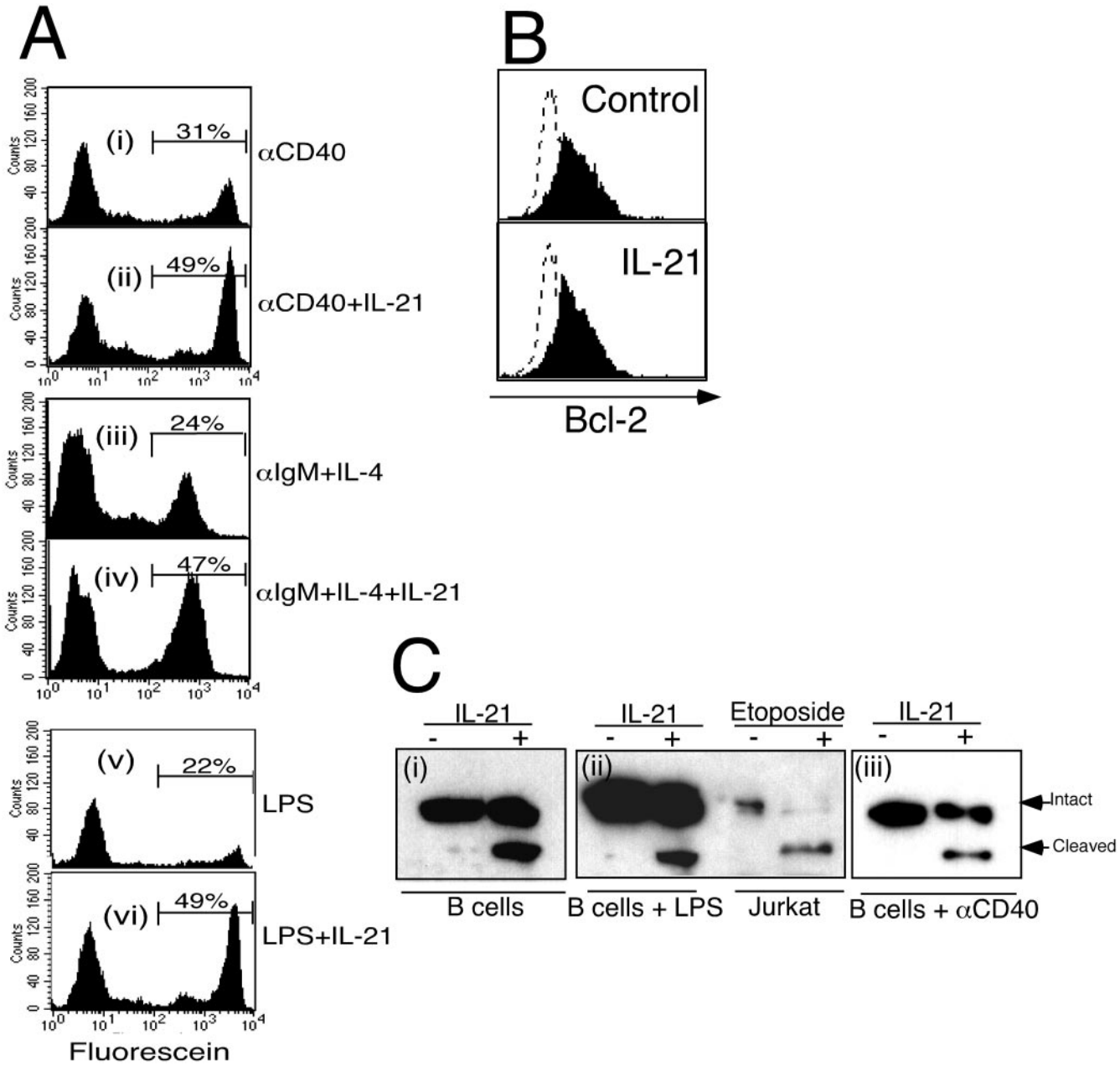

FIGURE 6. Effect of IL-21 on death and BCL2 expression in B cells. *A,* IL-21 augments DNA fragmentation (measured by TUNEL staining) in B cells stimulated with anti-CD40, anti-IgM + IL-4, or LPS. *Upper*, *middle*, and *lower pairs* of *panels* correspond to cells treated with anti-CD40 with or without IL-21 for 15 h, anti-IgM + IL-4 with or without IL-21 for 48 h, and LPS with or without IL-21 for 15 h, respectively. *B,* Bcl-2 levels were equivalent in untreated and IL-21-treated B cells. WT B cells were cultured with or without IL-21 for 24 h and intracytoplasmic Bcl-2 protein levels were evaluated by FACS analysis. *C,* Caspase activation by IL-21 as indicated by cleavage of poly(ADP-ribose) polymerase (PARP), a caspase substrate. Purified B cells were not activated or activated with LPS or anti-CD40, with or without IL-21, for 6 h. Cell lysates (50 μg) were electrophoresed and Western blotted with an anti-PARP Ab. The uncleaved and cleaved forms of PARP are also shown in Jurkat T cells treated with etoposide as a positive control.

IL-4. Only a fraction of the surface IgG1$^+$ cells expressed Syndecan-1 (Fig. 5*D*), indicating that IL-21 increased postswitch cells as well as plasma cells. For these experiments, we used culture conditions similar to those reported to allow apoptotic effects of IL-21 (8). Indeed, we independently demonstrated the apoptotic effects of IL-21 in combination with LPS, anti-IgM, or anti-CD40 (Fig. 6*A*). Interestingly, whereas this apoptosis is correlated with IL-21-mediated down-regulation of Bcl-2 mRNA levels (8), Bcl-2 cytoplasmic protein levels did not change in response to IL-21 (Fig. 6*B*), suggesting that other caspase-related mechanisms are more important (Ref. 8 and Fig. 6*C*), given the ability of IL-21 to induce cleavage of a caspase substrate (Fig. 6*C*). Thus, conditions that allow IL-21-mediated apoptosis also allow IL-21 to potently induce the maturation of stimulated B cells to postswitch cells and plasma cells in vitro and in vivo. Because we used purified B cells for the in vitro experiments, at least part of the effect of IL-21 on B cell differentiation is a direct effect.

IL-21 induces expression of Syndecan-1, Blimp-1, and Bcl-6, but it inhibits expression of Pax5

To elucidate the mechanism of the effect of IL-21 on B cell maturation and plasma cell differentiation, we examined the effects of IL-21 on B cell differentiation factors Blimp-1, Bcl-6, and Pax5. Blimp-1 is a transcription factor that has been identified as a master regulator of plasma cell differentiation (23), whereas Bcl-6 and Pax5 are required for germinal center formation (20). Interestingly, Blimp-1 and Bcl-6 can each inhibit expression of the other protein, and Blimp-1 additionally is an inhibitor of the expression of Pax5 (20, 24). We examined expression of these proteins in BCL1 3B3 cells, a B cell lymphoma cell line in which treatment with IL-2 and IL-5 can induce differentiation into Ig-secreting cells (25). In these cells, IL-21 induced expression of Syndecan-1 (Fig. 7*Ai*), whereas it decreased expression of MHC class II (Fig. 7*Aii*), consistent with plasma cell differentiation (13, 23). Analogous to the effect of IL-21 on CD23 expression in splenic B cells (Fig. 1*B*), IL-21 also decreased CD23 expression in BCL-1 cells (Fig. 7*Aiii*). Moreover, based on real-time PCR analysis, IL-21 induced expression of mRNA for both Blimp-1 and Bcl-6, whereas it inhibited expression of Pax5 mRNA (Fig. 7*B*, *i, ii,* and *iii*, respectively). The induction of Blimp-1 was at least as potent as that seen with the combination of IL-2 and IL-5, the "classical" stimulus for Blimp-1 in these cells (25); as expected, the combination of IL-2 and IL-5 inhibited expression of Bcl-6. The induction of both Blimp-1 and Bcl-6 by IL-21 was confirmed in purified splenic B cells. Induction was not seen in cells treated with anti-IgM alone, but the addition of IL-21 induced Blimp-1 protein expression as confirmed by Western blotting (Fig. 7*C*) and Blimp-1- and Bcl-6 DNA-binding activities, as evaluated by EMSAs (Fig. 7, *D* and *E*).

*IL-21 is elevated in the BXSB-*Yaa *autoimmune mouse model of SLE*

Given the ability of IL-21 to promote plasma cell differentiation, we speculated that this cytokine might contribute to humoral autoimmune processes. We therefore examined the BXSB-*Yaa*

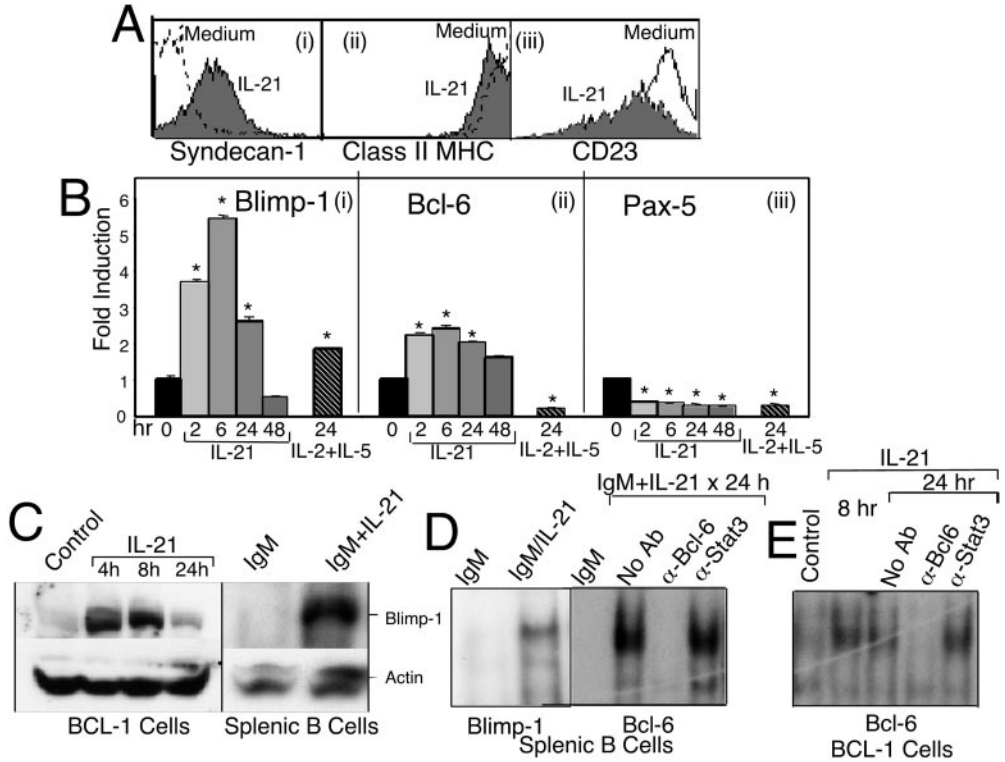

FIGURE 7. IL-21 increases Blimp-1 and Bcl-6 expression but diminishes Pax5. *A,* IL-21 induces Syndecan-1 expression (*i*), but diminishes MHC II and CD23 expression (*ii* and *iii*, respectively) in BCL-1 cells. *B,* IL-21 induces Blimp-1 (*i*) and Bcl-6 (*ii*), but decreases expression of Pax5 (*iii*) mRNA, as evaluated by real-time PCR in BCL-1 cells. The effect of IL-2 + IL-5 on expression of each gene is also shown. Error bars indicate the SD of replicate samples. Where not visible, the SD merged with the bars. *, $p < 0.01$ for induced vs uninduced mRNA levels. *C,* Induction of Blimp-1 protein, as evaluated by Western blotting in BCL-1 cells with IL-21 alone and in purified splenic B cells treated for 24 h with the combination of anti-IgM + IL-21 but not with anti-IgM alone. *D,* IL-21-mediated induction of Blimp-1 and Bcl-6 DNA-binding activities in splenic B cells as evaluated by EMSAs. Splenic B cells were isolated and treated with anti-IgM with or without IL-21 as described in *Materials and Methods*, and then Blimp-1 and Bcl-6 DNA-binding activities were evaluated using specific DNA probes. An Ab to Bcl-6 abolished the complex formed with the Bcl-6-binding probe, whereas an Ab to Stat3, which can bind the same probe (14), did not. *E,* IL-21 induction of Bcl-6 DNA-binding activity in BCL-1 cells.

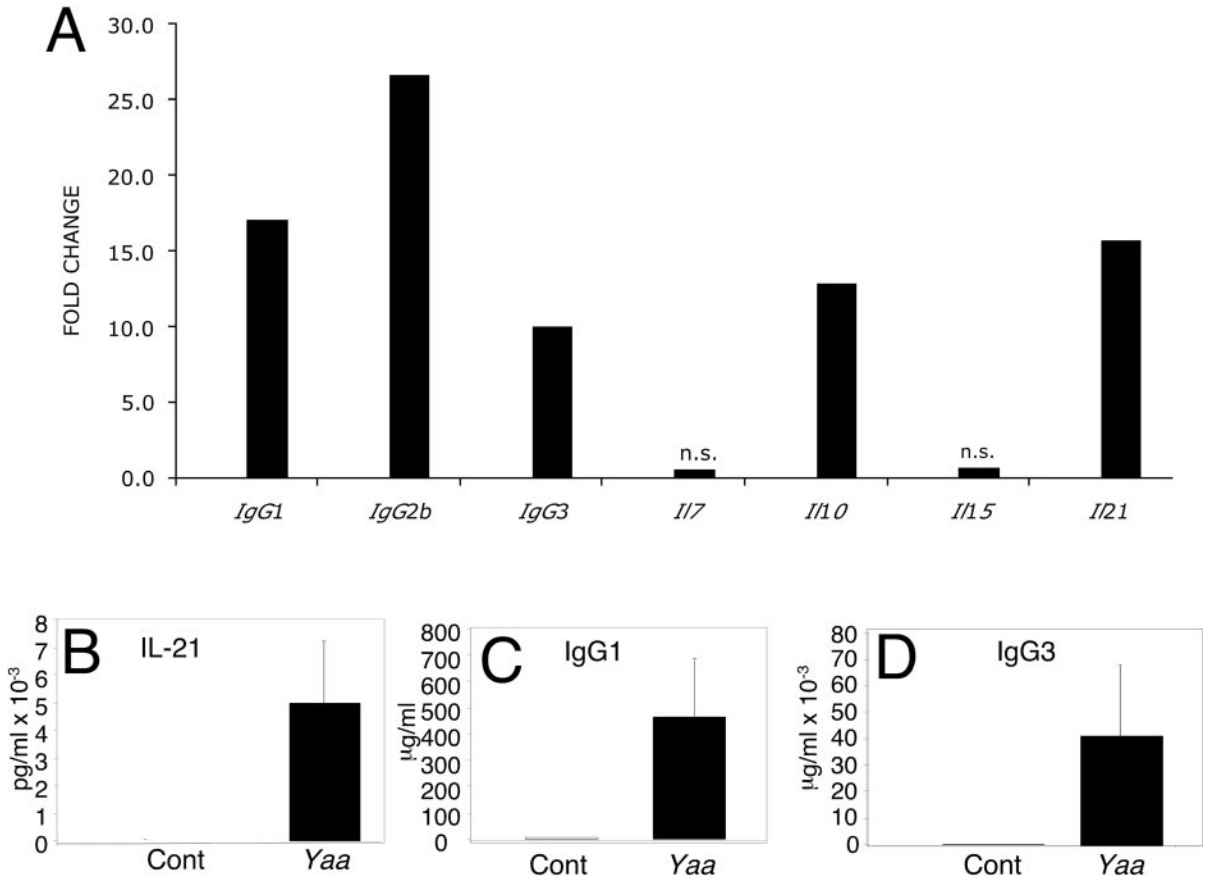

FIGURE 8. Markedly elevated IL-21 and IgG1 isotype expression in the BXSB-*Yaa* mouse. *A,* Quantitative PCR (QPCR) analysis of three BXSB-*Yaa* compared with three BXSB-*Yaa*$^+$ 16-wk-old male mice. Of 231 expressed genes, 9 genes were differentially expressed (at $p < 0.05$) such that they exceeded the 0.400 GPR cutoff, and they had the following rank order: *IgG2b* ($p < 0.02$), *Il10* ($p < 0.02$), *IgG1* ($p < 0.03$), *Il21* ($p < 0.03$), *DNase1* ($p < 0.04$), *IgG3* ($p < 0.04$), *Pdcd1* ($p < 0.05$), *Hsp70–1* ($p < 0.05$), and osteopontin ($p < 0.02$), with *IgG2b* being the most highly induced. The fold expression changes for *IgG2b*, *Il10*, *IgG1*, *Il21*, and *IgG3* as well as *Il7* and *Il15* are shown based on the normalizer gene, 18S RNA. Similar results including elevated *Il21* expression were obtain in two additional experiments. The genes for other cytokines that were examined, including *Il1b, Il2, Il4, Il5, Il6, Il12p35, Il12p40, Il18, Il24,* and *Il25* did not manifest significant expression changes. mRNAs that are elevated in a small number of cells or that are present at relatively low abundance may not be detected in this assay, which further underscores the significance of the genes whose expression was induced. Although these animals clearly have plasma cells and hypergammaglobulinemia, the proportions of these cells in the whole spleen is presumably small, as classic markers of these cells, including XBP-1, CD-9, and Blimp-1 were not elevated. *B–D,* Markedly elevated levels of serum IL-21 (*B*), IgG1 (*C*), and IgG3 (*D*) in BXSB-*Yaa* mice, as determined by double Ab sandwich ELISA. Shown are means ± SEM. The serum IL-21 levels in male BXSB-*Yaa* mice ranged between 62 and 13,900 pg/ml, whereas it was not detected in BXSB female mice. Serum IgG1 ranged between 16 and 2,000 μg/ml for BXSB-*Yaa* males, whereas the BXSB female controls had ~3.2 μg/ml; serum IgG3 ranged between 80 and a remarkable 50,000 μg/ml for BXSB-*Yaa* males, whereas the BXSB female controls had ~30 μg/ml.

mouse model, which is characterized by severe SLE, with lymphadenopathy, splenomegaly, leukocytosis, hypergammaglobulinemia, and severe immune complex-mediated glomerulonephritis, often with a nephrotic syndrome (26). Although the basis for the disease is unknown, severe SLE is dependent on the mutant Y chromosome-linked autoimmune accelerator *Yaa* locus. In contrast, mice carrying a C57BL/6 (B6)-derived WT *Yaa* allele exhibit a slowly developing chronic form of SLE (26). The mechanism mediating the action of the *Yaa* locus has been unclear.

Examination of splenocytes for expression of multiple genes revealed a striking age-dependent increase in *Il21* mRNA levels in BXSB-*Yaa* mice, compared with BXSB-*Yaa*$^+$ WT male mice (Fig. 8*A*; see also legend to Fig. 8). Interestingly, the expression of the genes encoding IL-7 and IL-15, two other γ_c-dependent cytokines, did not differ significantly in BXSB-*Yaa* and BXSB-*Yaa*$^+$ WT mice (Fig. 8*A*). Like *Il21*, *Il10* mRNA levels were also elevated in the BXSB-*Yaa* mouse (Fig. 8*A*). Corresponding to the increase in IL-21 mRNA, IgG1, IgG2b, and IgG3 mRNA levels were significantly elevated in BXSB-*Yaa* mice (Fig. 8*A*), as were serum levels of IL-21, IgG1, and IgG3 (Fig. 8, *B–D*). Thus, in addition to elevated Ig levels, among cytokines examined, we found a substantial increase in IL-21 in this model of SLE, suggesting that elevated levels of this cytokine may be involved in the pathogenesis of this disease.

Discussion

IL-21 is a pleiotropic cytokine with actions on T, B, and NK cells. To our knowledge, IL-21 is the only type I cytokine that can induce apoptosis of resting naive B cells, whereas other γ_c family cytokines are typically antiapoptotic. In the context of Ag activation, IL-2 can promote T cell death via a process known as activation-induced cell death (27). However, IL-21 is different in that it is proapoptotic for B and NK cells instead of T cells (Ref. 8 and our unpublished data), and whereas activation-induced cell death requires a prior activation signal, IL-21-induced apoptosis does not. In addition to apoptotic effects on mature B cells, we show that IL-21 can induce the accumulation of transitional B cells in the periphery. This could reflect physiological compensation for the

IL-21-mediated reduction in peripheral mature B cells, the ability of IL-21 to increase maturation and/or survival of immature B cells from the bone marrow, and/or an ability of IL-21 to interfere with signals that promote differentiation from transitional to mature B cells.

IL-21 TG mice exhibited elevated serum IgM and IgG1 and had increased surface $IgG1^+$ B cells in the spleen, suggesting that IL-21 could promote Ig isotype switching in vivo. A recent report demonstrates the ability of human IL-21 to promote isotype switching in CD40-activated human B cells (28). However, our data demonstrate that IL-21 can induce isotype switching and plasma cell differentiation in naive B cells before an encounter with secondary T cell signals, suggesting a role for IL-21 in early B cell responses to Ag. Strikingly, IL-21 down-regulated CD23 expression on mature B cells and promoted differentiation to Ig-secreting plasma cells both in vivo and in vitro. This contrasts to IL-4, which inhibits plasma cell differentiation (29) and induces CD23 expression on mature B cells (30). The ability of IL-21 to increase Ig-secreting cells clarifies why IgG1 Ab-forming cells are greatly decreased in *Il21r* knockout (KO) mice and why all Ig classes are diminished in *Il21r*/*Il4* double KO mice (6). Interestingly, the accumulation of surface $IgG1^+$ cells in response to IL-21 was reduced in the presence of IL-4, whereas the induction of Syndecan-1^+ cells was not altered. Thus, some but not all of the effects of IL-21 on the B cell immune response can be modulated by IL-4, consistent with our finding in *Il21r* KO vs *Il21r*/*Il4* double KO mice (6). The observation that IL-21 has antiapoptotic effects on some myeloma cell lines (31) is consistent with our observations that IL-21 is an inducing factor for plasma cells.

In summary, we have now elucidated some of the complex actions of IL-21 in the B cell immune response. IL-21 confers an apoptotic signal either on naive B cells or, alternatively, when bystander B cells are stimulated in an Ag-nonspecific manner by activated T cells via CD40 signaling (Ref. 8 and our unpublished observations). Following the initiation of a B cell immune response, this apoptotic signal of IL-21 might help to eliminate "bystander" B cells responsible for the nonspecific hypergammaglobulinemia that is initially observed. However, in B cells activated by B cell receptor signaling, our results indicate that IL-21 can enhance Ig production, isotype switching, and plasma cell production. We hypothesize that the effect of IL-21 on plasma cell differentiation results from its ability to increase Blimp-1 expression, whereas the induction of Bcl-6 may be important for subsequent differentiation of germinal center cells into postswitch cells. The IL-21-mediated down-regulation of Pax5 may bias responses toward plasma cell differentiation, as Pax5 is known to inhibit plasma cell differentiation but is thought to be required for memory cell maturation (20). Interestingly, IL-21 induced both Blimp-1 and Bcl-6, which exert mutually antagonistic effects (20, 24). Moreover, we demonstrate an increase in IL-21-mediated DNA-binding activity for both of these proteins, whereas no other known stimulus has been shown previously to activate the binding activity of both of these critical transcription factors. Such unprecedented actions help to explain how IL-21 can drive differentiation of B cells both into postswitch cells as well as to plasma cells. This may be another indication of the overall potency of IL-21 in stimulating multiple aspects of the differentiation of B cells, with its potentially having differential effects in a context-regulated manner.

Finally, although the genetic mutation and molecular basis for autoimmunity in the BXSB-*Yaa* mouse remains unknown, the possibility that elevated IL-21 accounts for the hypergammaglobulinemia and class switching to IgG isotypes characteristic of BXSB-*Yaa* mice is intriguing. This is consistent with the fact that the SLE-like autoimmune disease in these animals is dependent on $CD4^+$ T cells (32), the major source of IL-21 (1, 2). It is of interest that increased expression of IL-21 mRNA has been detected in autoimmune NOD mice (33). Overall, our data support critical roles for IL-21 in apoptosis, B cell differentiation, and plasma cell generation and suggest that this cytokine may be critically involved in the pathogenesis of autoimmune disease.

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