Deficiency of the Cyclin Kinase Inhibitor p21(WAF-1/CIP-1) Promotes Apoptosis of Activated/Memory T Cells and Inhibits Spontaneous Systemic Autoimmunity

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Abstract
A characteristic feature of systemic lupus erythematosus is the accumulation of activated/memory T and B cells. These G0/G1-arrested cells express high levels of cyclin-dependent kinase inhibitors such as p21, are resistant to proliferation and apoptosis, and produce large amounts of proinflammatory cytokines. Herein, we show that ablation of p21 in lupus-prone mice allows these cells to reenter the cell cycle and undergo apoptosis, leading to autoimmune disease reduction. Absence of p21 resulted in enhanced Fas/FasL-mediated activation-induced T cell death, increased activation of procaspases 8 and 3, and loss of mitochondrial transmembrane potential. Increased apoptosis was also associated with p53 up-regulation and a modest shift in the ratio of Bax/Bcl-2 toward the proapoptotic Bax. Proliferation and apoptosis of B cells were also increased in p21−/− lupus mice. Thus, modulation of the cell cycle pathway may be a novel approach to reduce apoptosis-resistant pathogenic lymphocytes and to ameliorate systemic autoimmunity.

Key words: CDKI • lupus • replicative senescence • Fas/FasL • cell cycle

Introduction
Foreign antigen-directed immune responses typically involve a sequence of T and B cell activation, clonal expansion, differentiation into effector cells, and, to maintain homeostasis, apoptosis (1, 2). The fraction (~5%) that escapes apoptosis constitutes the self-renewing, long-lived memory population. Autoimmune responses are not as clearly defined in this regard, although similar principles seem to govern antigen presentation, costimulation, and cytokine requirements (3). However, differences in the kinetics and homeostatic mechanisms must exist because self-antigens are continually present, and alterations in lymphocyte functions and phenotypes are observed with autoimmune disease progression.

A common feature of lupus-prone mice is the marked accumulation of activated/memory phenotype (CD44hi) CD4+ T cells resistant to proliferation and apoptosis (4, 5). Notably, such cells are arrested in the G0/G1-phase of the cell cycle and express high levels of certain cyclin-dependent kinase inhibitors (CDKIs), including p21WAF-1/CIP-1 (5), characteristics that are also associated with replicative senescence (6–8). We hypothesized (5) that repeated stimulation of self-reactive T cells might lead to a state akin to “replicative senescence,” wherein T cells no longer cycle, but persist and transcribe autoimmunity-promoting genes such as those encoding proinflammatory cytokines (9). A similar accumulation of activated B cells resistant to proliferation and apoptosis is also found in lupus-prone strains (10–13).

Although cell cycle and apoptosis are opposing biologic phenomena, studies have shown that they are interconnected (14, 15). TCR-, mitogen-, and superantigen-mediated apoptosis of mature T cells requires an initial progression through several cellular divisions (16–18). Furthermore, blocking cyclin B with antisense (AS) oligonucleotides inhibits TCR signal-mediated apoptosis (19). Thus, cell cycle blockade at the G0/G1-phase could be a major factor in apoptosis resistance and accumulation of activated/memory phenotype T cells in systemic autoimmunity.

Cell cycle progression is controlled by several proteins, including cyclins, cyclin-dependent kinases (CDKs), and CDKIs (15, 20). CDKIs are negative regulators of cyclin–
CDK complexes and, based on structural and functional characteristics, have been grouped into two distinct families, Ink4 and Cip/Kip (20, 21). The Ink4 proteins (p16INK4A, p15INK4B, p18INK4C, and p19INK4D) form binary complexes with CDK4 and CDK6 and block the G1 to S phase transition. In contrast, the pancyclin Cip/Kip proteins (p21CIP-1, p27KIP1, and p57KIP2) bind to the entire cyclin/CDK holoenzymes, inhibiting transitions at all stages of the cell cycle. Among the CDKIs, p21 is likely to play a prominent role in both cell cycle and apoptosis by promoting G1-arrest, inhibiting proliferating cell nuclear antigen, affecting key players in the apoptotic machinery (such as p53 and procaspase 3), and contributing to cellular senescence (21–24). The induction of p21 by several growth factors and cytokines, including IFN-γ, has also been reported (25, 26). Surprisingly, despite the multiple roles ascribed to this CDKI and the wide range of cells expressing this gene during development and cellular activation (21), p21-deleted mice develop normally at least up to 7 mo of age (27).

In light of this, we reasoned that absence of p21 might release repeatedly activated, self-reactive T and B cells from their replication/apoptosis-resistant state, allowing their entry into the S phase and subsequent apoptosis, thus reducing their accumulation and presumptive pathogenicity in systemic autoimmunity. Herein, we report that homozygous deletion of the p21 gene indeed reduced serological, cellular, and histologic disease manifestations and increased survival of male BXSB lupus-prone mice. This resistance to autoimmunity appeared to be primarily due to an increased susceptibility of activated/memory phenotype T and B cells to activation-induced cell death (AICD).

Materials and Methods

**Mice.** p21−/− mice, obtained from P. Leder (Harvard Medical School, Boston, MA), were backcrossed to the BXSB strain. Only male p21+/+ and p21−/− littermates were compared with survival, serologic, and histopathologic data compiled from mice at generations 7–11, and in vitro data from generations 10–13.

**Flow Cytometry.** Cells were stained with antibodies to CD4, CD8, CD11b, CD19, CD20, CD21, CD23, CD25, CD27, CD44, CD69, IgM, IgD, Annexin V, Fas, FasL, TCRα/β, IFN-γ, or PI (all obtained from BD Biosciences). Data were acquired on a FACS Calibur™ and analyzed with CELLQuest™ software (Becton Dickinson).

**Proliferation and Apoptosis Assays.** In vitro studies were conducted with cells from 1–2–mo-old mice, at an age at which frequencies and phenotypes of T and B cell subsets were equivalent between the two genotypes. LN cells were incubated with 5 µg/ml of soluble anti-CD3 and increasing concentrations of plate-bound anti-CD3 (BD Biosciences) for 48 h. [3H]Thymidine incorporation was measured 15 h later. Subsequently, the optimum coating concentration was selected (10 µg/ml of anti-CD3), and LN cells were plated on anti-CD3–coated plates plus 5 µg/ml of soluble anti-CD28, and analyzed for [3H]thymidine incorporation every 24 h for 6 d. B cells were activated with 10 µg/ml of soluble goat F(ab′)2 anti–mouse IgM (Jackson ImmunoResearch Laboratories) and IL-4. [3H]Thymidine incorporation was measured every 24 h for 3 d. In vivo proliferation of splenic T and B cells was determined by long-term bromodeoxyuridine (BrdU) incorporation (29). In brief, BrdU was administered in drinking water for 9 d (0.8 mg/ml), made fresh daily. After BrdU labeling, splenocytes were analyzed by FACS® using the BrdU Flow kit (BD Biosciences) according to the manufacturer’s instructions.

To assess T cell AICD, LN cells were cultured for 48 h with 0.5 µg/ml of soluble anti-CD3 and relighted with 10 µg/ml of plate-bound anti-CD3 (BD Biosciences; reference 18). To block AICD, anti-FasL antibody (BD Biosciences) or soluble Fas/Fc (a gift from D. Green, La Jolla Institute for Allergy and Immunology, San Diego, CA) was added at 10 µg/ml, whereas anti-Fas antibody (BD Biosciences) was added at 5 µg/ml to induce AICD. For B cell apoptosis, splenocytes were incubated with 10 µg/ml of soluble goat F(ab′)2 anti–mouse IgM.

T and B cells undergoing apoptosis were stained at 24-h intervals with either anti-CD4, anti-CD8, or anti-CD19, plus Annexin V and PI. The percentage of Annexin V+/PI− T or B cells was determined by FACS®. Loss of mitochondrial transmembrane potential was determined using the JC-1 mitochondrial transmembrane potential (ΔΨm) detection kit (Cell Technology, Inc.) according to the manufacturer’s instructions. Conversion of procaspases 8 and 3 to active caspases was assessed by the APO LOGIX carboxyfluorescein caspase detection kit and APO ACTIVE 3 antibody detection kit (Cell Technology, Inc.), respectively.

**RNase Protection Assay.** RNase protection assay of p21 expression on sorted CD19+ B cells was performed as described previously (5). In brief, riboprobes for p21 and L32 (housekeeping gene) were prepared and labeled with α-32P[UTP (Ribo probe System; Promega). Purified probes were hybridized to 5 µg of total B cell RNA (RPA Kit I; Torrey Pines Biolabs), protected products were run on a 6% polyacrylamide sequencing gel, and bands were revealed by overnight exposure on autoradiographic film (Eastman Kodak Co.).

**Stem and T Cell Cycling.** Bone marrow cells from 1–mo-old and LN T cells from 3–mo-old male BXSB p21+/+ or p21−/− mice (n = 4 mice/group) were stained with either a mouse lineage panel and anti–Sca-1 (both obtained from BD Biosciences), or anti-CD4 and anti-CD44. Cells were analyzed by FACS® after surface immunophenotyping and sequential incubation with 1.67 µM of DNA-binding dye Hoechst 33342 (Molecular Probes) and 1 µg/ml of RNA-binding dye Pyronin Y (Sigma-Aldrich; reference 30).

**AS Assays.** LN cells from 2–mo-old wild-type BXSB male mice were activated with plate-bound anti-CD3 or relighted to induce apoptosis, as aforementioned, in the presence of 400 nM of either of two Penetra tin–1–coupled p21-specific phosphorothioated AS oligonucleotides: p21AS no. 1, 5′-ACATCAC-CAGGATGGGACAT-3′ (31); and p21AS no. 2, 5′-TGTCAG-GCTGGTCTGCTCC-3′ (32) or a similarly processed control oligonucleotide obtained from Qbiogene: control AS, 5′-TG-GATCGCATGTCGAG-3′ (32).

**Western Blots.** Wild-type and p21−/− BXSB T cells were activated with 10 µg/ml anti-CD3 plus 5 µg/ml anti-CD28 and analyzed for p21, Bax, Bcl-2, and p53 protein expression. In brief, lysates were prepared from 2.0 × 106 cells, and protein was measured by microprotein assay (Bio-Rad Laboratories). Proteins were separated on a 15% SDS-PAGE (Bio-Rad Laboratories), transferred to polyvinyl difluoride membrane (Immobilon–P, Millipore), and blocked with 5% milk powder in PBS. After overnight incubation at 4°C with primary antibodies to p21, Bax, Bcl-2, or p53, proteins were revealed with appropriate horseradish peroxidase–conjugated secondary antibodies (BD Biosciences or Santa Cruz Biotechnology, Inc.) followed by SuperSignal.
Results

To examine the role of p21 in systemic autoimmunity, we generated congenic p21−/− lupus-susceptible BXSB mice and assessed disease manifestations, immune homeostasis, and the responses of T and B cells. Males were exclusively studied because severe early life lupuslike disease in this strain requires the Yaa (Y chromosome accelerator of autoimmunity and lymphoproliferation) susceptibility gene (13). Reduced Hypergammaglobulinemia and Autoantibodies in p21−/− BXSB Mice. Initial studies were performed to determine the effects of p21 deficiency on autoimmune manifestations. Control mice exhibited typical hypergammaglobulinemia at 4 mo of age, whereas all IgG subclasses were significantly lower in p21−/− littermates, approaching levels seen in C57BL/6 normal mice (Fig. 1 a). Antichromatin levels, predominantly of the IgG2a subclass, were also high in the wild-type littermates. In contrast, antichromatin autoantibodies of all subclasses were greatly reduced in the p21−/− mice (Fig. 1 b).

Enhanced Survival and Reduced Kidney Disease in p21−/− BXSB Mice. The mortality and immunopathology of the control p21+/+ litters were similar to those of our male BXSB colony, indicating sufficient backcrossing of the major BXSB lupus susceptibility genes. In contrast, there was a dramatic reduction in mortality and GN in BXSB p21−/− mice. Control BXSB males showed 50% mortality at 5.6 mo and 100% mortality by 6.3 mo, whereas ~80% of p21−/− mice were alive at 13 mo and ~65% at 16.5 mo (Fig. 1 c). GN (2.1 ± 0.4 vs. 3.4 ± 0.2; P < 0.05), IgG deposit scores (1.5 ± 0.2 vs. 3.1 ± 0.4; P < 0.05) (Fig. 1 d), and blood urea nitrogen levels (1.1 ± 0.1 vs. 3.2 ± 0.2; P < 0.05) were also significantly reduced in 4-mo-old BXSB p21−/− mice. Thus, p21 is clearly essential for the accelerated lupus in BXSB mice.

Fever Total and Activated/Memory T and B Cells in p21−/− BXSB Mice. The effects of p21 deficiency on in vivo autoimmune T and B cell homeostasis, proliferation, and apoptosis were examined. The weights and cellular compo-

**Figure 1.** Decreased autoimmune disease in male p21−/− BXSB mice. (a and b) Reduced serum polyclonal (left) and antichromatin (right) IgG subclasses in 4-mo-old male BXSB p21−/− mice (n = 8 mice/group; mean ± SEM). (shaded bars) BXSB p21−/− mice. (unshaded bars) p21+/+ BXSB mice. For all polyclonal and antichromatin total and subclass IgG levels, P < 0.05 for p21−/− versus p21+/+. (c) Increased cumulative survival rates of p21−/− BXSB mice (P < 0.0001). Male BXSB p21+/+(n = 16) and p21−/− (n = 15) littermates were followed for up to 500 d. (D) BXSB p21−/− mice. (○) BXSB p21+/+ mice. (d) Glomerular pathology (top) and IgG deposits (bottom) of representative 4-mo-old p21+/+ and p21−/− mice. Increased segmental mesangial proliferation and accumulation of periodic-acid Schiff-positive mesangial matrix material were seen in p21−/− mice, whereas p21−/− mice exhibited significantly less glomerular damage, as well as decreased segmental granular mesangial and capillary wall deposits of IgG (top, 630×; bottom, 400×).
sion of spleen and LN are shown in Table I. Spleen weight and total cell numbers as well as numbers of CD8+ and activated/memory CD4+CD44hi T cells were unaffected by the lack of p21, but, strikingly, total splenic CD4+ and activated/memory CD4+CD44hi cells were significantly reduced (P < 0.05). When spleen cells were examined for preapoptotic cells, p21−/− mice had a significant twofold increase in the number of Annexin V−binding CD4+CD44hi T cells, which represented a fourfold increase in percentage (16.3 ± 2.1 vs. 4.0 ± 0.8%; P < 0.05; Table I). In contrast, splenic B cells (CD19+), activated (Table I, CD69+), memory (CD27+), B1a (CD5+), follicular, and marginal zone B cell populations (not depicted) were unaffected. BXSB male mice also develop an expanded peripheral blood population of unusual Mac-1+ MHC class II− monocytes whose contributions to disease remains unknown (35). However, the frequency of these cells was equivalent in p21−/− and p21+/+ mice (20.3 ± 1.3 vs. 19.2 ± 2.1%).

For the LN, there were significant approximately fivefold reductions in both weight and total cell numbers in p21−/− mice at 4 mo as well as lower numbers of T and B cells and their subsets (Table I, P < 0.05). Notably, the percentage of preapoptotic (Annexin V+) activated/memory CD4+CD44hi T cells was ~2.6-fold higher in the p21−/− versus p21+/+ mice (21.5 vs. 8.2%; P < 0.05). The absolute number of intracellular IFN-γ+ T cells was also greatly reduced in LNs of p21-deficient mice (Table I).

Enhanced Cycling of Activated/Memory CD4+CD44hi T Cells in p21−/− BXSB Mice. Analysis of in vivo proliferation indicated enhanced cycling of CD4+ T cells in p21-deficient animals, with significant increases in both total

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**Table I.** Spleen and LN Analysis of BXSB p21−/− Mice

| T cells (× 10^6) | B cells (× 10^6) |
|------------------|------------------|
|                  |                  |
| **Spleen**       |                  |
| BXSB p21+/+      |                  |
| 0.4 ± 0.05 *     | 190.4 ± 21.0     |
| 29.4 ± 4.4       | 24.4 ± 3.3       |
| 0.97 ± 0.02 (%)  | 8.8 ± 4.6        |
| CD4+             | ND               |
| CD4+CD44hi       | 15.3 ± 8.0       |
| CD4+CD44hiAV     | 14.5 ± 1.5       |
| CD4+IFN-γ+       | 6.7 ± 0.4        |
| CD8+             | ND               |
| CD8+CD44hi       | 102.7 ± 20.5     |
| CD8+CD44hiAV     | 6.0 ± 1.3        |
| CD19+            | 126 ± 14.4       |
| CD19+CD69hi      | 9.1 ± 2.2        |
| p21−/−           |                  |
| 0.3 ± 0.1        | 170.4 ± 37.1     |
| 18 ± 2.2b        | 11.7 ± 1.4b      |
| 1.91 ± 0.03% (16%) | 6.7 ± 0.4        |
| CD4+             | ND               |
| CD4+CD44hi       | 14.5 ± 1.5       |
| CD4+CD44hiAV     | 6.7 ± 0.4        |
| CD19+            | ND               |
| CD19+CD69hi      | 102.7 ± 20.5     |
| **LN**           |                  |
| BXSB p21+/+      |                  |
| 0.5 ± 0.11       | 249.2 ± 21.0     |
| 75.6 ± 9.9       | 45.1 ± 6.4       |
| 3.7 ± 0.2 (8%)   | 14.8 ± 2.6       |
| CD4+             | ND               |
| CD4+CD44hi       | 22.2 ± 5.3       |
| CD4+CD44hiAV     | 14.8 ± 2.6       |
| CD19+            | 2.2 ± 0.6        |
| CD19+CD69hi      | 117.8 ± 2.5      |
| p21−/−           |                  |
| 0.1 ± 0.05b      | 52 ± 4.7b        |
| 15.3 ± 5.2b      | 6.5 ± 0.2b       |
| 1.4 ± 0.1 (22%)  | 4.5 ± 1.5b       |
| CD4+             | 3.7 ± 1.0b       |
| CD4+CD44hi       | 17 ± 0.4b        |
| CD4+CD44hiAV     | 0.4 ± 0.1b       |
| CD19+            | 27.2 ± 4.1b      |
| CD19+CD69hi      | 0.8 ± 0.03b      |

Spleen and combined LN (axillary, inguinal, cervical, and mesenteric) weights (n = 8 mice/group) are shown (mean ± SEM g). T and B cell subsets from p21+/+ and p21−/− BXSB mice at 4 mo of age are indicated as total numbers (mean ± SEM) of splenocytes or their respective subset (n = 5 mice/group).

| Table II. Long-Term In Vivo BrdU Incorporation of T and B Cells in Male BXSB p21+/+ and p21−/− Mice |
|--------------------------------------------------|
| Cell population | BXSB p21+/+ | BXSB p21−/− |
|------------------|------------|------------|
|                  | %          | %          |
| CD4+             | 8.9 ± 0.9  | 19.0 ± 2.3 |
| CD4+CD44hi       | 24.2 ± 2.0 | 36.5 ± 3.2 |
| CD8+             | 9.7 ± 1.2  | 15.2 ± 2.4 |
| CD8+CD44hi       | 13.1 ± 1.8 | 12.4 ± 2.9 |
| CD45R/B220+      | 25.5 ± 2.1 | 29.0 ± 5.8 |

4-mo-old male BXSB p21+/+ and p21−/− were fed BrdU in drinking water for 9 d. T and B cells (B220+) defined by FACS as BrdUhi are indicated as percentages of the listed cell populations.

*P < 0.05 between BXSB p21+/+ and p21−/− mice (n = 3).
were stimulated with increasing concentrations of plate-bound anti-CD3 antibodies and assessed for [3H]thymidine incorporation at the n 0.5m mice. (c) Increased AICD of p21 AS or control oligonucleotides (all AS oligonucleotides at 400 nM) plus 5 μg/ml of soluble anti-CD28 antibodies in the presence of either of two p21-specific AS oligonucleotides, but not with a control oligonucleotide, showed significantly increased proliferation (Fig. 3 d) and apoptosis (Fig. 3 e), approaching the levels of the p21−/− cells (P < 0.05). Similar results were observed with CD8+ T cells (unpublished data). The oligonucleotides had no discernible effect on viability, and Western blot analysis confirmed that the two specific p21 AS oligonu-
cleotides (400 nM; n = 3 mice/group). (■) AS oligo no. 1. (□) AS oligo no. 2. (▲) Control oligonucleotide. (e, inset) Western blot analysis of wild-type BXSB T cells treated with either p21 AS or control oligonucleotides. T cells were stimulated with 10 μg/ml anti-CD3 and 5 μg/ml CD28 antibodies in the presence of AS or control oligonucleotides (400 nm). Whole cell lysates were analyzed by Western blot using anti-p21 and antiactin antibodies. *, P < 0.05 by Student’s t test.
Untreated (Fig. 4 a). Accordingly, kinetic studies showed that the frequency of p21-deficient T cells (n = 3 mice/group) were first stimulated with 10 μg/ml of soluble anti-CD3 and 5 μg/ml CD28 for 48 h and then with 5 μg/ml anti-Fas antibody and analyzed for percentage of apoptotic (Annexin V+PI+) CD4+ T cells. (b) BXSB p21−/− mice. (c) BXSB p21−/- mice, * P < 0.05. (b) Increased caspase 8 and 9 activation and reduced mitochondrial membrane potential (ΔΨm) in p21−/− T cells after induction of AICD. T cells (n = 3 mice/group) were stimulated with 0.1 μg/ml of soluble anti-CD3, retigulated with 10 μg/ml of plate-bound anti-CD3, and analyzed by FACs® for activation of caspases 8 and 9 and for change in mitochondrial transmembrane potential. The percentage of cells expressing activated caspases 8 or 9 and reduced ΔΨm is indicated (mean ± SEM). P < 0.05 between p21+/+ and p21−/− for all time points shown. (c) Fas blockade inhibits AICD of p21+/+ and p21−/− T cells. T cells (n = 3 mice/group) were stimulated with 0.1 μg/ml of soluble anti-CD3 for 48 h, retigulated with 10 μg/ml of plate-bound anti-CD3 in the presence or absence of 10 μg/ml of blocking anti-FasL antibody, and analyzed for Annexin V positivity. Similar inhibition was also observed after treatment with Fas-blocking soluble Fas/Fc. (●) BXSB p21+/+ mice plus anti-FasL. (▲) BXSB p21+/+ mice plus anti-FasL. (●) BXSB p21+/+ mice. (▲) BXSB p21−/− mice. *, P < 0.05 for untreated (● or ▲) versus anti-FasL–treated groups (● or ▲) at 48 and 72 h, and for untreated p21−/− (●) versus p21+/+ (▲) mice at 72 h.

Enhancement of the Extrinsic Pathway of Apoptosis in p21−/− T Cells. Fas and FasL expression of anti-CD3- and anti-CD28–activated p21+/+ and p21−/− CD4 T cells was equivalent (unpublished data). However, anti-Fas–induced apoptosis of activated CD4 T cells was higher in p21-deficient T cells (Fig. 4 a). Accordingly, kinetic studies showed that the frequency of p21−/− T cells undergoing AICD that had converted initiator procaspase 8 and effector procaspase 3 to active caspases was significantly higher than wild-type cells at all time points (Fig. 4 b). Similarly, loss of mitochondrial transmembrane potential was more pronounced in p21−/− than p21+/+ cells (Fig. 4 b). However, AICD was inhibited in both types of T cells by either anti-FasL mAb (Fig. 4 c) or Fas/Fc (not depicted). Thus, as expected, the Fas/FasL pathway is the primary mediator of AICD in p21+/+ and p21−/− T cells, but the CD95 signaling cascade and associated events are amplified in p21−/− cells.

Participation of the Intrinsic Pathway of Apoptosis. Although the extrinsic CD95–mediated apoptosis pathway is generally sufficient for AICD, depending on cell type and/or signal strength, the intrinsic pathway may facilitate this process (38–40). p21−/− T cells cultured with anti-CD3 and anti-CD28 for 48 h expressed 1.9-fold more p53 and 1.7-fold less Bcl-2 protein than control cells. Although Bax expression was unaltered, the ratio of Bax/Bcl-2 shifted moderately toward the proapoptotic Bax (5.3 in p21−/− vs. 3.1 in p21+/+) (Fig. 5; P < 0.05). In addition, activated wild-type T cells cultured in the presence of transfecting p21 AS oligonucleotides showed a 1.7-fold decrease in Bcl-2 and, in this case, a 1.9-fold increase in Bax expression, resulting in a more pronounced shift in the Bax/Bcl-2 ratio (8.1; P < 0.05).

Enhanced Proliferation and Apoptosis of p21−/− B Cells. As reported for T cells, B cells from lupus-predisposed mice have also been shown to be both arrested in G1 and apoptosis resistant (10, 41). Indeed, we detected high p21 levels in male, but not female, B cells (Fig. 6 a). Accordingly, activation with anti-IgM plus IL-4 induced higher proliferation (Fig. 6 b; P < 0.05), and cross-linking with anti-IgM resulted in accelerated and enhanced apoptosis of p21−/− compared with wild-type B cells (Fig. 6 c; P < 0.05).
Fewer Quiescent Stem Cells in p21<sup>−/−</sup> BXSB Mice. When TD antibody responses to TNP-KLH were examined, p21<sup>−/−</sup> mice showed fivefold higher primary IgG (Fig. 6 d) and marginally increased IgM (not depicted) responses at day 14 compared with wild-type mice. However, 7 d after challenge, the secondary IgG response increased twofold in p21<sup>−/−</sup> versus 12-fold in the wild-type mice. Notably, the secondary response in the p21<sup>−/−</sup> mice precipitously declined thereafter to almost baseline levels by day 35, whereas the decline was gradual in the wild-type mice, a finding compatible with enhanced AICD in the p21<sup>−/−</sup> mice.

Figure 6. Enhanced proliferation and apoptosis, and reduced late secondary immunoglobulin responses of p21<sup>−/−</sup> B cells from male BXSB mice. (a) Increased p21 expression in B cells from older male BXSB mice. Sorted B cells (CD19<sup>+</sup>) from 3-mo-old wild-type male or female BXSB mice (n = 5 mice/group) were analyzed by RNase protection assay for expression of p21 and L32 (control). (b) Increased proliferation of p21<sup>−/−</sup> B cells after IgM cross-linking. Splenocytes (n = 3 mice/group) were stimulated with 10 μg/ml of soluble goat F(ab')<sub>2</sub> anti-mouse IgM in the presence of IL-4 and assessed for [3H]thymidine incorporation (mean ± SEM cpm). (●) BXSB p21<sup>−/−</sup> mice. (○) BXSB p21<sup>+/+</sup> mice. (c) Enhanced AICD of p21<sup>−/−</sup> B cells. Annexin V positivity of B cells was assessed after anti-IgM stimulation with 10 μg/ml of soluble goat F(ab')<sub>2</sub> anti-mouse IgM (n = 3 mice/group). (●) BXSB p21<sup>−/−</sup> mice. (○) BXSB p21<sup>+/+</sup> mice. (d) Anti-TNP antibody levels after primary and secondary immunizations. 2-mo-old male BXSB p21<sup>−/−</sup> and p21<sup>+/+</sup> mice (n = 4 mice/group) were injected s.c. with 100 μg TNP-KLH emulsified in CFA. Secondary responses were assessed by boosting mice s.c. with 100 μg TNP-KLH in saline on day 21. Mice were bled at the indicated times, and serum was analyzed by ELISA. (●) BXSB p21<sup>−/−</sup> mice. (○) BXSB p21<sup>+/+</sup> mice. * P < 0.05 by Student's t test.

Discussion

Herein, we demonstrate that deletion of the CDKI p21 significantly reduced serologic, cellular, and histologic disease manifestations and increased survival of lupus-prone BXSB mice. Furthermore, there was reduced accumulation of proliferation- and apoptosis-resistant T and B cells through a novel mechanism involving enhanced entry of these cells into the cell cycle followed by apoptotic death. These findings clearly show that accumulation of replication/apoptosis-resistant T and B cells in this spontaneous lupus model is dependent on increased p21 expression, and suggest that these cells contribute significantly to the autoimmune and inflammatory processes that are critical for disease pathogenesis.

Classically, p21 inhibits cell cycle entry by blocking formation of active cyclin–CDK complexes. However, more recently identified p21 functions, including inhibition of DNA replication through proliferating cell nuclear antigen binding, repression of E2F, interference with c-Myc, control of certain transcription coactivators, and other interactions may also be involved in cell cycle blockade (21, 24).

Growth factors initiate and maintain the entry of cells from G<sub>0</sub> to S phase in the cell cycle (42). Mitogen-activated protein kinase (MAPK) signaling induces D cyclins, resulting in activation of CDK4 and CDK6, and progression of cells through G<sub>1</sub>. However, MAPK signaling has also been shown to induce CDKIs, including p21, and growth arrest (43–45). Thus, it has been suggested that strong or sustained activation of MAPK signaling leads to induction of CDKIs and cell cycle arrest, whereas transient activation promotes cell cycle.

This cellular activation model provides a possible explanation for the accumulation of activated/memory phenotype CD4<sup>+</sup> T cells in lupus (Fig. 7). We hypothesize that genetic susceptibility predisposes lupus T cells to hyperrespond through a variety of mechanisms, such as increased antigen presentation or lack of regulatory signaling. Many of these mechanisms have been revealed recently by analyses of spontaneous as well as gene knockout and transgenic mice with lupuslike disease (46, 47). This enhanced activa-
data indicate that the fraction of activated/memory CD4+ T cells that escape AICD in wild-type autoimmune mice (because of increased CDKIs) do not accumulate in G0/G1-phase in p21-deficient mice; instead, they proliferate and become susceptible to Fas-mediated apoptosis. Thus, the lack of G1-arrest and increased apoptosis of B cells, in agreement with studies showing diminished this G0/G1 arrested state, and their accumulation. It is significant that despite the multiple pancyclin inhibitors (p21, p27, and p57), deletion of p21 can accomplish this effect, implying that functional redundancy among these molecules is only partial.

Extensive data have shown previously that p21 inhibits apoptosis of various cell types by directly affecting expression/function of several molecules involved in this process (24, 52). We demonstrated that p21 deletion amplified T cell AICD by promoting the extrinsic pathway of apoptosis. Thus, in the absence of p21, Fas/FasL-mediated apoptosis was enhanced, concurrent with increased conversion of procaspases 8 and 3 to active caspases, and loss of mitochondrial transmembrane potential. Moreover, wild-type T cells behaved similarly to p21-deficient cells when transfected with p21 AS oligonucleotides. The results clearly establish that p21 is a significant inhibitor of Fas/FasL-mediated T cell apoptosis. Enhanced sensitization of glioma cells to CD95-mediated apoptosis with p21 AS oligonucleotides associated with increased caspase 8 and 3 activation has been shown previously (40). We also observed increased apoptosis of anti-IgM cross-linked p21−/− B cells, in agreement with studies showing diminished G1-arrest and increased apoptosis of B cells treated with p21 AS oligonucleotides (53).

A potential biochemical explanation for the increased activation of procaspase 3 may be provided by the series of observations by Suzuki et al., who reported that p21 released from the CDK4/6 complex by survivin translocates to the mitochondria, binds to a putative mitochondrial adaptor protein, and sequesters procaspase 3 (54–57). Moreover, because p21 binds to the cleavage site of procaspase 3, conversion to the p17 active fragment is also reduced, thus impeding Fas-mediated apoptosis. In addition, evidence has been presented that active caspase 3 can cleave p21, reducing its inhibitory effects and accelerating apoptosis (58–61). Increases in proximal caspase 8 activation of p21-deficient cells undergoing AICD might be attributed to less interference with procaspase 8 cleavage (24), or to a feedback effect mediated by the increased levels of activated caspase 3 (40).

Depending on cell type and/or signal intensity, the extrinsic and intrinsic pathways of apoptosis may converge (38, 39). We found that activated p21−/− T cells had only a moderate increase in p53 levels and a slight shift in the ratio of Bax/Bcl-2 toward the proapoptotic Bax. Stabilization of p53 and inversion of the Bcl-2/Bax ratio has also been reported in carcinoma cells undergoing chemotherapeutic drug-induced apoptosis (62). However, it appears that the intrinsic pathway plays a minor role, if any, in the enhanced AICD of p21−/− T cells.

A slight reduction in the percentage (7.8 vs. 12.2%) of quiescent G0-phase hematopoietic stem cells in p21−/− BXSb mice was also observed. However, this was not associated with hematopoietic precursor insufficiency, consistent with a previous paper in which p21−/− bone marrow cells that required three serial passages before significant stem cell deficiency was observed (30). Furthermore, the reduction of T and B cells was limited to only certain lymphoid organs and cell subsets. Thus, it is highly unlikely that the slight decrease in percentage of quiescent stem cells plays a significant role in the reduction in activated/memory T and B cells and autoimmune disease in p21−/− mice.

One of the most striking findings in this work is the difference in TD antibody responses of p21−/− and wild-type BXSb mice. In the primary response, p21−/− mice showed
much higher IgG levels, whereas in the secondary response, the antibody levels declined more rapidly compared with wild-type mice. The enhanced primary antibody response in p21\(^{-/-}\) mice is likely due to accelerated proliferation of helper T cells, whereas the rapid reduction in the secondary response is likely caused by increased AICD. This finding is compatible with our hypothesis that, under conditions of sustained T cell stimulation by constant exposure to self-antigens, lack of p21 leads to enhanced proliferation and apoptosis of self-reactive helper T cells, thereby reducing autoantibody responses.

Our findings contrast sharply with an initial report that older female, but not male, p21\(^{-/-}\) mice of mixed 129/Sv \(\times\) C57BL/6 (129 \(\times\) B6) background develop severe lupuslike features (36). Subsequent analyses by us with a different group of p21\(^{-/-}\) mixed-background 129 \(\times\) B6 mice and, more significantly, with female BXS B p21\(^{-/-}\) mice containing all lupus-predisposing genes except the Yaa, showed no appreciable induction of autoimmune disease (34, 37). Several papers have documented that the 129 \(\times\) B6 mixed genomes spontaneously develop signs of systemic autoimmunity with low levels of GN, especially in females of advanced age (37, 63–66). Therefore, systemic autoimmunity in gene-deleted 129 \(\times\) B6 mice should be carefully controlled by performing a sufficient number of backcrosses to attain genetic homogeneity, and should include wild-type littermates. The present paper makes it clear that modulation of the cell cycle pathway by deleting the CDKI p21 inhibits the development of systemic autoimmunity.

This is further supported by our recent finding that deficiency in another CDKI (i.e., p27) also results in reduction of lupuslike disease in male BXSB mice (unpublished data).

Although our work focused on male BXSB lupus mice, expansion of activated/memory phenotype T cell populations is common to other lupus strains (5, 67), and is observed in human systemic lupus erythematosus (68, 69). Accumulation of G1/G0-arrested T cell populations has also been observed in other autoimmune diseases, such as rheumatoid arthritis (70) and insulin-dependent diabetes (71, 72), and even in aging (73), suggesting that these cells may also contribute to the pathogenesis of these diseases and to immune senescence.

The present paper raises the possibility that efforts to block the activity of CDKIs may be a means to intervene in systemic autoimmunity and other immune-related disorders. The primary contribution of p21 in disease pathogenesis, and the relatively benign consequences of p21 deficiency (24), makes it a particularly promising therapeutic target. Blockade of p21 and possibly other CDKIs could be a novel approach that, instead of inhibiting, promotes proliferation and hence apoptosis of the accumulated autoreactive T and B cells. This may be a particularly powerful strategy for eliminating disease-promoting cells in advanced autoimmune diseases.

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