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 serologic, 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−/− littersmates 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, B220, CD21, CD23, CD25, CD11b, 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 (1 μCi) 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 (28). 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 for every 4 d 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 FACSA® 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 repleted 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 FACSA®. 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 (Riboprobe 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 FACSA® 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 repleted to induce apoptosis, as aforementioned, in the presence of 400 nM of either of two Penetratin–1–coupled p21-specific phosphorothislated AS oligonucleotides: p21AS no. 1, 5′-ACATCAC-CAGGATGGACAT-3′ (31); and p21AS no. 2, 5′-TGTCAG- GCTGGTCCTGCCCTCC-3′ (32) or a similarly processed control oligonucleotide obtained from Qbiogene: control AS, 5′-TG- GATCCGACATGTCAGA-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 × 107 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.
chemiluminescence (Pierce Chemical Co.). Radiographs were scanned on the Personal Densitometer SI and analyzed by ImageQuant 4.2a (both Amersham Biosciences).

T-dependent (TD) Responses. Mice were injected s.c. with 100 μg trinitrophenyl (TNP)–keyhole limpet hemocyanin (KLH; Biosearch Technologies) emulsified in CFA. Secondary responses were assessed by a s.c. boost of 100 μg TNP-KLH in saline on day 21. Mice were bled at the indicated times, and antibody levels were determined by ELISA (33).

Serologic Analysis. Total and antichromatin serum IgG subclasses were measured by ELISA using 96-well plates coated with goat anti–mouse IgG (Jackson ImmunoResearch Laboratories) or mouse chromatin (29). Bound IgG subclasses were detected using AP-conjugated IgG subclass–specific antibodies (Caltag). Standard curves for each subclass were generated using calibrated mouse serum (Accurate Chemical and Scientific Company).

Kidney Disease. AZOSTIX strips (Bayer) were used to measure blood urea nitrogen and graded on a 1–4 scale (1 = 5–15, 2 = 15–26, 3 = 30–40, and 4 = 50–90 mg/dl). Histologic examination of periodic-acid Schiff–stained kidneys was done in a blinded manner at 4 mo of age, and severity of glomerulonephritis (GN) was defined on a scale of 0–4 (34). For immunohistology, frozen kidney sections were fixed in ice-cold acetone, blocked, and incubated with anti–IgG-FITC (Vector Laboratories); deposits were scored as described previously (29).

Statistics. The Student's t test was used for group mean comparisons, and survival was analyzed by the Kaplan–Meier method with comparisons by log-rank test. P-values <0.05 were considered significant.

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. 1a). 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. 1b).

Enhanced Survival and Reduced Kidney Disease in p21−/− BXSB Mice. The mortality and immunopathology of the control p21+/+ littermates 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 all IgG subclasses were significantly lower in p21−/− mice (Fig. 1b). p21+/+ BXSB mice. (a and b) Reduced serum polyclonal (left) and antichromatin (right) IgG subclasses in 4-mo-old male BXSB p21−/− mice (mean ± SEM). (shaded bars) BXSB p21−/− mice. (unshaded bars) BXSB p21+/+ 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. (E) 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×).
Table I.  Spleen and LN Analysis of BXSB p21+/− Mice

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<tr>
<th></th>
<th>Weight (× 10^6)</th>
<th>T cells (× 10^6)</th>
<th>B cells (× 10^6)</th>
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<tr>
<td></td>
<td></td>
<td>CD4⁺</td>
<td>CD4⁺CD44hi</td>
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<tr>
<td>Spleen BXSB p21+/−</td>
<td>0.4 ± 0.05</td>
<td>190.4 ± 21.0</td>
<td>29.4 ± 4.4</td>
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<tr>
<td>Spleen BXSB p21+/−</td>
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<td>170.4 ± 37.1</td>
<td>18 ± 2.2b</td>
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<tr>
<td>LN BXSB p21+/−</td>
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<td>249.2 ± 21.0</td>
<td>75.6 ± 9.9</td>
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<tr>
<td>LN BXSB p21+/−</td>
<td>0.1 ± 0.04b</td>
<td>52.4 ± 4.7b</td>
<td>15.3 ± 3.2b</td>
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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).

aPercent of CD4⁺CD44hi T cells that are AV⁺.

bP < 0.05 between p21+/+ and p21−/− mice. ND, not determined.

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.

aP < 0.05 between male BXSB p21+/+ and p21−/− mice (n = 3).

Figure 2.  Representative distribution of G0/G1 versus G2/M phase in CD4⁺CD44hi T cells. Splenocytes from 3-mo-old mice (n = 4 animals/group) were stained with antibodies to CD4 and CD44, followed by sequential incubation with Hoechst 33342 (DNA-binding dye) and Pyronin Y (RNA-binding dye) and analyzed by flow cytometry. Pyronin Y staining of G0/G1-phase CD4⁺CD44hi T cells from p21+/− and p21+/+ BXSB mice is shown (P < 0.05).
Figure 3. Enhanced proliferation and apoptosis of p21−/− T cells from male BXSB mice. (a) Increased proliferation of p21−/− T cells after in vitro cross-linking of antigen receptors. LN cells (n = 3 mice/group) were stimulated with increasing concentrations of plate-bound anti-CD3 (0.1–20 μg/ml) plus 5 μg/ml of soluble anti-CD28 antibodies and assessed for [3H]thymidine incorporation at 72 h (mean ± SEM cpm). (shaded bars) BXSB p21−/− mice. (unshaded bars) p21+/+ BXSB mice. (b) Kinetics of in vitro proliferation. LN cells (n = 3 mice/group) were stimulated with 10 μg/ml of plate-bound anti-CD3 plus 5 μg/ml of soluble anti-CD28 antibodies and assessed for [3H]thymidine incorporation at the indicated time points. Results are representative of two independent experiments (n = 3 mice/group). (●) BXSB p21−/− mice. (○) BXSB p21+/+ mice. (c) Increased AICD of p21−/− T cells. T cell were stimulated with 0.5 μg/ml of soluble anti-CD3 for 48 h followed by TCR reengagement with 10 μg/ml of plate-bound anti-CD3 (n = 3 mice/group). Percent of apoptotic (Annexin V+/PI−) CD4+ T cells was determined by FACS®. Similar results were obtained with CD8+ T cells. (●) BXSB p21−/− mice. (○) BXSB p21+/+ mice. (d) Increased proliferation of wild-type BXSB T cells treated with p21 AS oligonucleotides. LN cells (n = 3 mice/group) were stimulated with 10 μg/ml of plate-bound anti-CD3 plus 5 μg/ml of soluble anti-CD28 antibodies in the presence of either p21 AS or control oligonucleotides (all AS oligonucleotides at 400 nM) and assessed for [3H]thymidine incorporation (mean ± SEM cpm). (e) Increased AICD of wild-type BXSB T cells treated with p21 AS oligonucleotides. Wild-type BXSB T cells were stimulated with 0.5 μg/ml of soluble anti-CD3 followed by TCR reengagement with 10 μg/ml of plate-bound anti-CD3 in the constant presence of p21 AS or control oligonucleotides (400 nM; n = 3 mice/group). (●) AS oligo no. 1. (○) AS oligo no. 2. (▲) Control oligonucleotide. (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.
cleotides, but not the control oligonucleotide, efficiently blocked p21 protein expression (Fig. 3 c, inset).

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 apoptotic 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−/− BXSB Mice. When TD antibody responses to TNP-KLH were examined, p21−/− 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 rechallenge, the secondary IgG response increased twofold in p21−/− versus 12-fold in the wild-type mice. Notably, the secondary response in the p21−/− 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−/− mice.

Enhanced Primary, but Reduced Late Secondary, TD IgG Responses in p21−/− BXSB Mice. When TD antibody responses to TNP-KLH were examined, p21−/− 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 rechallenge, the secondary IgG response increased twofold in p21−/− versus 12-fold in the wild-type mice. Notably, the secondary response in the p21−/− 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−/− mice.

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 blockage (21, 24).

Growth factors initiate and maintain the entry of cells from G1 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 G1. 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+ 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 p21 appears to restore homeostasis of autoreactive CD4+CD44hi T cells by preventing their transition to a senescent-like state.

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).

Figure 7. T cell senescence and p21 in systemic autoimmunity. In lupus-prone mice, the accumulated activated/memory CD4+ T cells are in a state resembling replicative senescence. We hypothesize that repeated stimulation of CD4+ T cells (depicted by the hypothetical movement of cells from the memory to effector compartments) by self-antigens (Ag) leads to resistance to proliferation and apoptosis (senescent-like cell), due in large part to increased levels of CDKIs. Senescent cells are metabolically active and can produce proinflammatory cytokines. As shown in this work, the gradual accumulation of these activated/memory phenotype CD4+ T cells and subsequent development of autoimmunity is dependent on p21. Furthermore, our

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 slight reduction in the percentage (7.8 vs. 12.2%) of quiescent G0/G1-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 × C57BL/6 (129 × B6) background develop severe lupuslike features (36). Subsequent analyses by us with a different group of p21−/− mixed-background 129 × B6 mice and, more significantly, with female BXSB 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 × 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 × 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 G0/G1-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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