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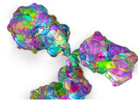

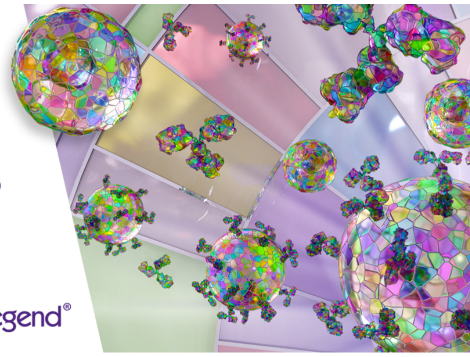

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p21$^{CIP1/WAF1}$ Controls Proliferation of Activated/Memory T Cells and Affects Homeostasis and Memory T Cell Responses[1]

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Development of autoantibodies and lupus-like autoimmunity by 129/Sv × C57BL/6 p21$^{-/-}$ mice has established that cell cycle deregulation is one the defective pathways leading to break of tolerance. Memory T cell accumulation is thought to be related to tolerance loss in murine lupus models. We studied T cell memory responses in C57BL/6 p21$^{-/-}$ mice that develop lupus-like disease manifestations. p21 did not affect primary proliferation of naive T cells, and was required for cycling control, but not for apoptosis of activated/memory T cells. When we induced apoptosis by secondary TCR challenge, surviving memory T cells depended on p21 for proliferation control. Under conditions of secondary T cell stimulation that did not cause apoptosis, p21 was also needed for regulation of activated/memory T cell expansion. The requirement for p21 in the control of T cell proliferation of activated/memory T cells suggests that in addition to apoptosis, cycling regulation by p21 constitutes a new pathway for T cell homeostasis. Concurring with this view, we found accumulation in p21$^{-/-}$ mice of memory CD4^{+} T cells that showed increased proliferative potential after TCR stimulation. Furthermore, OVA immunization of p21$^{-/-}$ mice generated hyperresponsive OVA-specific T cells. Overall, the data show that p21 controls the proliferation of only activated/memory T cells, and suggest that p21 forms part of the memory T cell homeostasis mechanism, contributing to maintenance of tolerance. *The Journal of Immunology,* **2007, 178: 2296–2306.**

One of the Cip/Kip family inhibitors is p21, best known for its role in cell cycle inhibition. It regulates early G_1-S transition by inhibiting cyclin-dependent kinases in complex with cyclins A and E or D (1). Although p21 deletion was predicted to lead to tumor development, it did not lead to spontaneous cancer in young mice (2, 3). The most striking phenotypic property conferred by p21 deficiency is severe lupus-like autoimmunity, glomerulonephritis, and death in mostly female 8- to 11-mo-old mixed background (129/Sv × C57BL/6), leading to death (4). To limit genetic background variances, all studied mice were derived from limited crosses of three breeding pairs (4). Nevertheless, given the multifactorial nature of lupus-like disease (5), we considered that a mixed background may enhance the autoimmunity effect conferred by p21 deficiency (4). It is now well established that hybrid (129/Sv × C57BL/6)F_1 or F_2 mice develop mostly humoral autoimmunity due to epistatic interactions between the 129/Sv and the C57BL/6 genome (6). Because the mixed background p21$^{-/-}$ mice exhibited dramatically enhanced symptoms of lupus, including lethal glomerulonephritis (4), in comparison with the established mild autoreactivity of 129/Sv × C57BL/6 mice (6), p21 deficiency appears as an essential autoimmunity enhancer.

Mixed background p21$^{-/-}$ mice (derived from an unspecified number of intercrosses) were analyzed in another study (7). p21 deficiency enhanced humoral responses, whereas glomerulonephritis development was mild and was not associated with gender differences. The authors of this work considered p21 deficiency as a minor autoimmunity player (7). An explanation for the results of this study could be that extensive intercrosses between mixed background p21$^{-/-}$ mice led to background heterogeneity and an eventual loss of autoimmunity-enhancing elements that are needed for severe autoimmunity in the absence of p21.

Further support for the role of p21 as a key autoimmunity enhancer was provided by the laboratory of A. Fornace Jr. (Harvard School of Public Health, Boston, MA), showing that p21 deficiency led to severe gender-linked autoimmunity and death (8). Furthermore, this group demonstrated that, introducing the p21 deletion to Gadd45a-deficient mice, which are also autoimmunity predisposed, dramatically increased autoimmunity and mortality in the doubly deficient mice (8). Therefore, the combination of the p21 deletion with certain elements of the 129/Sv × C57BL/6 background (4, 8) or the Gadd45a deletion (8) reveals that p21 deficiency may result in the induction of severe autoimmunity. p21 deficiency thus emerges as an autoimmunity accelerator that is background dependent, as are other principal autoimmunity accelerators, including the *lpr* or the *Yaa* genes (9).

In addition to the above studies, BXSB p21$^{-/-}$ mice were generated, and it was reported that p21 deficiency did not affect the mild autoimmunity that characterizes BXSB female mice (10). On the basis of this work, the authors concluded that deletion of p21 is unrelated to autoimmunity induction and claimed that previous

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data showing an autoimmune predisposition in p21$^{-/-}$ mice could be confounded by the autoimmunity predisposition of mixed background mice (10). There are several arguments against this consideration. First, the possibility that the mixed background can confound the effect of a deleted gene has been considered for genes encoded on the distal region of chromosome 1 or on chromosome 7, because interaction of 129 intervals on distal chromosome 1 or on chromosome 7 with the C57BL/6 genome leads to autoantibody production (6). This possibility is discarded due to the localization of p21 on chromosome 17. Second, the lethal autoimmunity effects of p21$^{-/-}$ mixed background mice (4, 8) are much more severe than the autoantibody production by mixed background mice (6). Finally, we have commented previously on the possible complications associated with the multifactorial nature of autoimmune disease (11). Distinct genetic defects can lead to autoimmune disease, and epistatic events between susceptibility loci may confuse the relevance of susceptibility loci (12). Therefore, the fact that p21 deficiency does not accelerate female BXSB autoimmunity could simply indicate that the BXSB background lacks the susceptibility alleles that could interact with the p21 deletion. Alternatively, however, a specific locus of the BXSB background may have a suppressive effect on the p21 deficiency-related events that would be otherwise sufficient for disease development.

In a related experiment with BXSB male mice that carry the *Yaa* gene, the same authors reported that p21$^{-/-}$ BXSB male mice developed reduced autoimmunity (13). Because p21 deficiency did not affect female BXSB disease, the reduced disease of p21$^{-/-}$ BXSB male mice appears to be the result of an interesting interaction between the *Yaa* gene or its effects and p21 deletion. On the basis of this disease reduction in p21$^{-/-}$ BXSB male mice, it was proposed that blocking cell cycle inhibitor activity could be considered a way to treat autoimmunity. This proposal, made on the basis of the effect of the p21 deficiency on the *Yaa* gender-dependent BXSB autoimmunity model (13) and without considering the severe lupus development by p21-deficient mice in conjunction with the Gadd45a deletion or with the 129S/v × C57BL/6 background (4, 8), could be misleading. Indeed, in agreement with the view that p21 deficiency is indeed an autoimmunity inducer, it was shown recently that the inhibitory effect of p21 on cell cycle has a therapeutic effect on lupus disease in (New Zealand Black × New Zealand White)F_1 mice (14), providing evidence for a suppressor effect of p21 in lupus autoimmunity. Finally, our approach in the present study was to introduce the p21 deletion on the autoimmunity-free C57BL/6 background with the intention of clarifying any doubts about the role of p21 in autoreactivity induction.

TCR-dependent stimulation of naive T cells leads to their activation and proliferation, followed by homeostasis and memory T cell generation (15, 16). Homeostasis regulates total lymphocyte number, the proportion of naive and memory T cells, and the extent of T cell memory expansion. Homeostasis is based on the balance between growth and death of immune cells (17); apoptosis defects could thus lead to defective homeostasis and T cell accumulation, causing break of tolerance (17, 18). Deregulation of the T cell cycle by lack of p21 leads to tolerance loss (4, 7, 8). Association of T cell tolerance and cell cycle regulation was verified in mice deficient in other cell cycle-associated molecules such as the p53 effector gene Gadd45a (8) and E2F2 (19). These data confirmed our hypothesis that, in addition to apoptotic defects, deficient cell cycle control could be determinant for loss of tolerance and autoimmunity development (20). T cell proliferation anomalies were also shown to be associated to diabetes development (21, 22).

In addition to its cell cycle regulatory role, p21 is proposed to act as an apoptosis regulator (23). The effect of p21 deficiency on apoptosis is debated, however; some studies report that p21 may inhibit apoptosis (13, 24), whereas others indicate that p21 promotes apoptosis (25, 26). These contrasting views might be explained by considering that the p21 effect on apoptosis may be cell type dependent.

Studies of naive T cells from three different studies (4, 8, 27) show that p21 deficiency does not influence primary TCR-dependent responses. In contrast, another study argued that p21$^{-/-}$ lymph node (LN)[3] T cells proliferate more than wild-type (wt) controls after primary T cell proliferation (7). In this work, however, instead of stimulating naive T cells, usually obtained from 8-wk-old mice, LN T cells from 4- to 6-mo-old T cells that presumably contain a rather elevated proportion of memory T cells were TCR stimulated. Therefore, the increased proliferation of post-TCR-challenged p21-deficient T cells may simply represent elevated responses in memory T cells and not enhanced primary cycling of naive T cells.

T cell proliferation during the initial IL-2 expansion phase after primary T cell activation is unaffected by p21 deficiency (4, 27). We nonetheless established that p21 is required for T cell proliferation during the late IL-2 expansion phase (4). Treatment with a p21 peptidyl mimic inhibits expansion of activated lymphocytes from lupus-prone (New Zealand Black × New Zealand White)F_1 mice (14). These findings suggest that p21 control of cycling may depend on T cell activation status. Other studies showed inhibition of T cell proliferation after transfection with p21 (28) or direct uptake of recombinant p21 protein (29), although T cell status is not discussed.

We explored the association between p21-dependent regulation of T cell proliferation and the activation and/or differentiation status of cycling T cells. We generated p21$^{-/-}$ C57BL/6 mice to minimize genetic background interference in the interpretation of results. Our data showed a critical role for p21 in regulating proliferation of TCR-stimulated activated/memory T cells. p21 did not affect Fas/Fas ligand (FasL)-mediated apoptosis, but governed cycling of apoptosis-resistant memory T cells; p21 may thus control homeostasis by regulating activated/memory T cell proliferation. Finally, general and specific T cell memory responses were increased in p21$^{-/-}$ mice. Overall, our study shows that p21 cycling regulates activated/memory T cell proliferation, which is reflected in p21-dependent memory T cell responses in mice.

Materials and Methods

Mice

Mice were kept in our animal facility under pathogen-free conditions. C57BL/6 mice were obtained from The Jackson Laboratory. Hybrid 129/Sv × C57BL/6 p21$^{-/-}$ mice were backcrossed to the C57BL/6 background for eight generations, and C57BL/6 p21$^{-/-}$ mice were obtained by intercrossing. C57BL/6 F8 p21$^{-/-}$ breeders were analyzed for 80 microsatellite markers (low-density analysis) (30), and showed >99% identity to the C57BL/6 background. Segments of chromosome 1 from a 129 mouse interact with components of the C57BL/6 background, giving rise to spontaneous mild autoimmune manifestations (6). High-density marker analysis of chromosome 1 (15 markers) confirmed that our p21$^{-/-}$ mice were of C57BL/6 origin (data not shown). The other disease-related locus linked to the 129 background was identified on the chromosome 7 15- to 26.5-cM region (6); analysis of this segment (5 markers) in our p21$^{-/-}$ mice verified their C57BL/6 origin (data not shown). Our institutional review committee reviewed and approved all mouse studies.

Tissue culture and assays

CD4$^+$ and CD8$^+$ cells were purified from spleen by negative selection using pan-B220 and anti-CD8 or anti-CD4 Dynabeads, respectively (Dynal Biotech), and showed >90% purity. CD4$^+$ cells (10^6/ml) were Con A

[3] Abbreviations used in this paper: LN, lymph node; AICD, activation-induced cell death; FasL, Fas ligand; PI, propidium iodide; wt, wild type.

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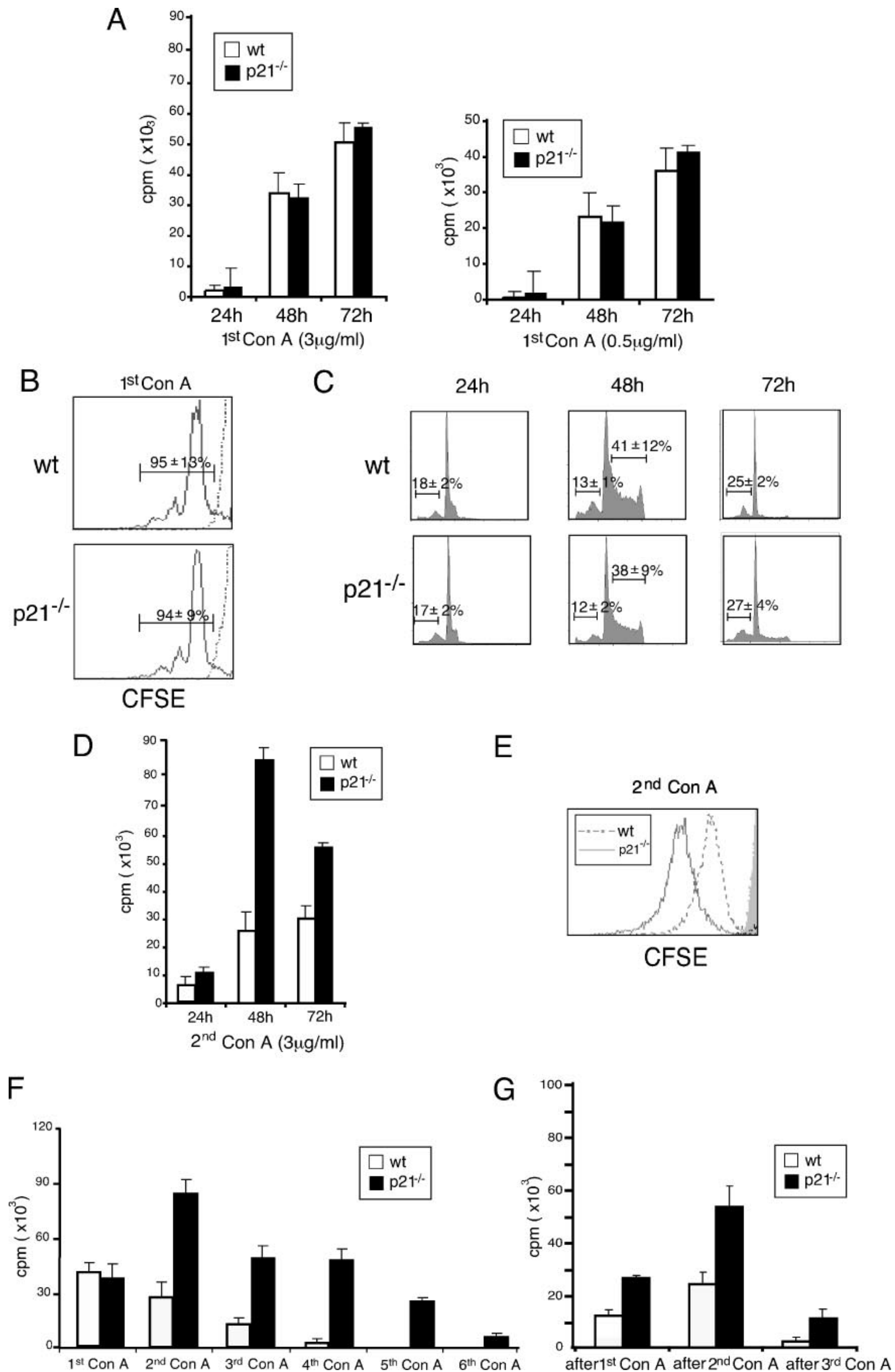

FIGURE 1. Increased proliferative responses of p21$^{-/-}$ T cells. *A*, CD4$^+$ splenocytes were stimulated with Con A at 3 or 0.3 μg/ml, and proliferation kinetics were determined by [^{3}H]thymidine incorporation. Values represent mean ± SD (n = 5). *B*, Dilution of fluorescence intensity in CFSE-labeled (live gated, PI-negative) CD4$^+$ T cells was evaluated by FACS analysis 72 h after primary (3 μg/ml) Con A stimulation. Solid lines represent CFSE dilution after stimulation, whereas broken lines represent fluorescence of CFSE-labeled, but not stimulated cells. Values represent the proportion of proliferating

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stimulated for 3 days (3 or 0.5 μg/ml; Sigma-Aldrich) in medium containing 10% IL-2. [^{3}H]Thymidine (1 μCi/100 μl) was added for the last 16 h of culture, and proliferation was measured. Anti-CD3 (BD Pharmingen) stimulations were also performed with 5 or 0.5 μg/ml. Con A-stimulated cells were washed and recultured in 10% IL-2 supernatant (6 days); cells were subcultured in fresh medium every 3 days. In the final IL-2 culture period, cells were restimulated with Con A. IL-2-treated cells were also restimulated in anti-CD3-coated plates (1 μg/well) with 10% IL-2, or with soluble anti-CD3 (1 μg/ml) and anti-CD28 Ab (0.5 μg/ml; both from BD Pharmingen); these anti-CD3 and anti-CD8 concentrations were also used for primary T cell stimulation. Caspase peptide benzyloxycarbonyl-Val-Ala-Asp (zVAD)-fmk was added to some cultures (100 μM final concentration) to inhibit apoptosis. Sorted $CD4^+CD44^{high}CD62L^{low}$ cells were Con A stimulated (3 μg/ml, 72 h) and tested for proliferation, as above.

$CD4^+$ T splenocytes from OVA (Sigma-Aldrich)-injected mice were purified using Dynabeads, as above, and OVA stimulated in vitro (100 μg/ml; 10^5 cells/ml; 24 h) in RPMI 1640 containing 10% FCS with irradiated splenocytes (0.5×10^6/ml). Proliferation was measured using [^{3}H]thymidine, as above.

For apoptosis induction, IL-2-expanded $CD4^+$ cells were treated in anti-CD3-coated plates (10 μg/well) or with the soluble human rFasL kit (500 ng/ml; ALX-850-014; Alexis).

Flow cytometry

$CD4^+$ and $CD8^+$ T cells were phenotyped by anti-CD4, anti-CD8, anti-CD44, and anti-CD62L Ab staining (all from BD Pharmingen); stained cells were analyzed on an LSR cytometer (BD Biosciences). For thymocyte analysis, anti-CD4 and anti-CD8 Abs were used. Cell cycle was analyzed by propidium iodide (PI) staining (20 μl/ml). Apoptosis levels were measured by TUNEL (Beckman Coulter). For proliferation analysis, cells were incubated with CFSE (2.5 μM; Molecular Probes) in PBS (37°C, 10 min), followed by 1-min incubation with 1:5 (v/v) FCS. Cells were washed twice with RPMI 1640/5% FCS. Cell surface protein expression and CFSE staining were monitored by flow cytometry and analyzed using the manufacturer's software.

In vivo BrdU administration

Mice were given BrdU (0.8 mg/ml; Sigma-Aldrich) in drinking water, prepared freshly every 2 days, for a 9-day period. BrdU expression was determined after triple staining, as described (21), using appropriate Abs and a FITC-conjugated anti-BrdU Ab (BD Biosciences).

Western blot

Cells were washed with PBS and treated with lysis buffer (1% Nonidet P-40, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 mM NaF, 1 mM PMSF in 50 mM Tris (pH 7.4)). Lysates were resolved in 12% SDS-PAGE and analyzed by Western blot using an anti-p21 Ab (Santa Cruz Biotechnology).

Histological and serological analysis

Histological examination of kidneys was done in a blind manner; glomerulonephritis severity was evaluated on H&E-stained paraffin sections using a $0-4^+$ scale (31). For immunohistochemistry, 6-μm frozen kidney sections were processed (31) and stained with FITC goat anti-mouse IgG (Southern Biotechnology Associates). Anti-DNA IgG isotypes and anti-OVA Ab were detected by ELISA. IgG isotype reactivity was normalized for equivalent OD against a myeloma isotype control (31) (Caltag Laboratories).

Statistics

The Mann-Whitney *U* test was used for all statistical analyses; p values <0.05 were considered significant.

Results

p21-dependent regulation of proliferation in repeatedly TCR-challenged T cells

To examine the role of p21 in the cycling regulation of activation/memory T cells, we used a procedure of repeated TCR stimulations separated by IL-2-dependent expansions, a protocol used to study apoptosis induction and generation of memory T cells. Naive $CD4^+$ $p21^{-/-}$ and $p21^{+/+}$ T cells were purified from spleens of 8- to 10-wk-old mice (used throughout the study unless otherwise specified). $CD4^+$ cells were Con A stimulated for 3 days and subsequently expanded with IL-2 for 6 days. Cells were then subjected to a second Con A stimulation. After primary stimulation, C57BL/6 $p21^{-/-}$ and wt T cells responded equally to TCR challenge, as measured by [^{3}H]thymidine uptake at 24, 48, and 72 h poststimulation (Fig. 1*A*, *left*), concurring with previous studies (4, 8, 27). To assure that the equal TCR response after primary stimulation was not concentration dependent, as was the case for $p27^{-/-}$ $CD8^+$ T cells (32), the assay was repeated with low Con A concentration and similar results were obtained for $p21^{-/-}$ and wt T cells (Fig. 1*A*, *right*). These data were corroborated by studies showing that, after primary stimulation, CFSE-labeled $p21^{-/-}$ and wt T cells showed equal dilution of fluorescence intensity, indicating that in both cases ~95% of the cells proliferated after 72 h after challenge (Fig. 1*B*). Primary stimulation with high (5 μg) or low (0.5 μg) concentrations of soluble anti-CD3 confirmed that proliferation after primary TCR stimulation was also p21 independent (data not shown). Because it has been reported that p21-deficient thymocytes resist Fas-induced apoptosis (25), we examined whether p21 could affect apoptosis of activated naive T splenocytes. Following primary Con A challenge, cell cycle analysis showed that p21 deficiency did not influence apoptosis induction of T cells at 24, 48, and 72 h poststimulation, nor the proportion of proliferating T cells as illustrated after exposure to Con A (Fig. 1*C*). We also examined T cell subpopulations by anti-CD4 and anti-CD8 staining in the thymus and found no differences between $p21^{-/-}$ C57BL/6 and wt mice (data not shown), in agreement with a previous report (32).

Following primary Con A stimulation, IL-2-dependent culture, and secondary TCR challenge, the large majority of T cells undergo apoptosis during the first 24 h (see below). T cells that survive apoptosis proliferate, as measured by [^{3}H]thymidine uptake at 48 and 72 h poststimulation. We observed a notable increase in $p21^{-/-}$ T cell proliferation compared with that of wt counterparts (Fig. 1*D*). These data were corroborated by studies showing that, 72 h after secondary stimulation, CFSE-labeled $p21^{-/-}$ T cells showed greater dilution of fluorescence intensity than controls (Fig. 1*E*). In this case, dilution of CFSE generated a single peak probably due to the fact that second Con A-treated T cells were previously proliferating and may have expanded in an almost synchronized fashion.

cells expressed as mean ± SD ($n = 4$). *C*, PI staining after 24, 48, and 72 h of primary Con A stimulation. In all cases, values represent the proportion of apoptotic T cells or cycling T cells expressed as mean ± SD ($n = 4$). *D*, $CD4^+$ splenocytes were stimulated with 3 μg/ml Con A, followed by a 6-day IL-2 treatment and a second Con A treatment, and proliferation kinetics were determined by [^{3}H]thymidine incorporation. *E*, Dilution of fluorescence intensity in CFSE-labeled (live gated, PI-negative) $CD4^+$ T cells 72 h after secondary Con A stimulation. The filled histogram represents the fluorescence of CFSE-labeled, but not stimulated, cells. Median fluorescence intensity values for $p21^{-/-}$, 140 ± 8 vs wt 417 ± 12 ($n = 4$; $p < 0.0003$). *F*, T cells were stimulated for five rounds of Con A, each followed by IL-2-dependent expansion. Each Con A stimulation was evaluated in parallel with primary stimulation of wt $CD4^+$ lymphocytes to confirm stimulation equivalence. Representative data are shown for one of five independent experiments. *G*, Degree of proliferation of cells cultured for 6 days with IL-2 after primary, secondary, and tertiary Con A treatments. For *F* and *G*, values represent mean ± SD ($n = 5$).

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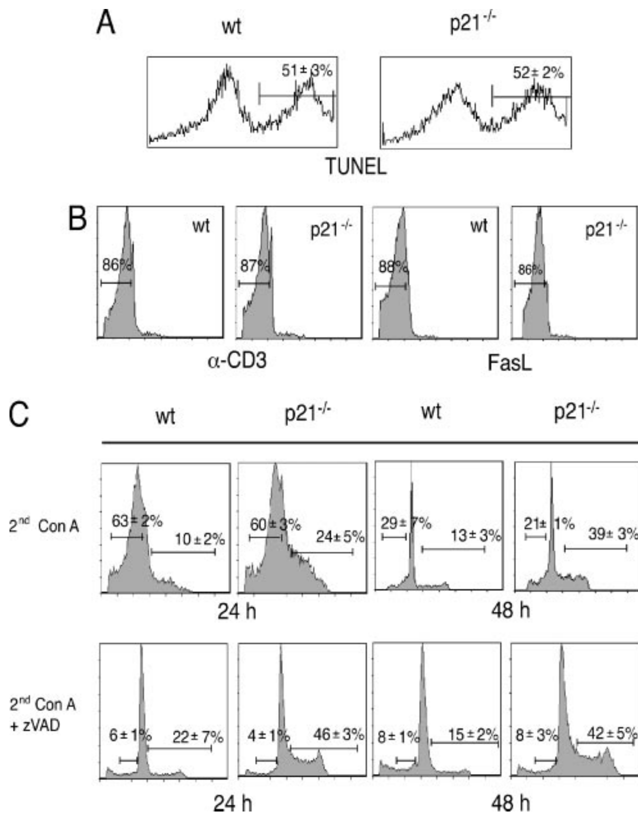

FIGURE 2. Apoptosis characteristics of $p21^{-/-}$ T cells. *A*, TUNEL analysis 24 h after secondary Con A stimulation of $CD4^+$ T cells. *B*, PI staining of T cells treated with anti-CD3 or FasL for 12 h. Proportions of apoptotic T cells correspond to one representative mouse of five tested. *C*, PI staining of secondary Con A-challenged T cells alone (*top*) or with zVAD (*bottom*). In all cases, values represent the proportion of apoptotic T cells or cycling T cells expressed as mean ± SD ($n = 4$).

The increased proliferation potential of $p21^{-/-}$ T cells was not confined to secondary TCR stimulation, because after IL-2 expansion and a third Con A challenge, $p21^{-/-}$ T cells proliferated at higher rates than controls (Fig. 1*F*). p21 deletion increased the potential for repeated stimulation, because $p21^{-/-}$ T cells proliferated after as many as five stimulation cycles, whereas wt T cells responded to no more than three Con A challenges (Fig. 1*F*). When stimulated with anti-CD3 and anti-IL-2, or with anti-CD3 and anti-CD28, $p21^{-/-}$ T cells maintained their hyperproliferative capacity compared with wt T cells (data not shown). We obtained similar results using $p21^{-/-}$ $CD8^+$ T cells (data not shown).

In 129/Sv × C57BL/6 mouse T cells, we previously showed that p21 influences proliferation during the late phase of IL-2 expansion, specifically at 6 days postprimary TCR stimulation (4); this effect was confirmed in this study (Fig. 1*G*). $p21^{-/-}$ T cell proliferation increased compared with wt controls during IL-2 expansions, not only after primary Con A stimulation, but also after secondary and tertiary TCR challenge (Fig. 1*G*).

Intact apoptosis in repeatedly activated $p21^{-/-}$ T cells

Secondary stimulation leads to apoptosis of a large proportion of T cells. We tested whether $p21^{-/-}$ T cell hyperproliferation was due to an increase in proliferating, apoptosis-resistant T cells caused by a p21 deficiency-associated apoptotic defect. This possibility appeared unlikely, because $p21^{-/-}$ T cells showed greater CSFE dilution than wt T cells after secondary TCR challenge (Fig. 1*E*), indicating that $p21^{-/-}$ T cells undergo a larger number of divisions. In addition, apoptosis was minimal during IL-2 expansion, with no differences between $p21^{-/-}$ and wt T cells (data not shown) (4), whereas p21 deficiency led to increased T cell proliferation during IL-2 treatment (Fig. 1*G*).

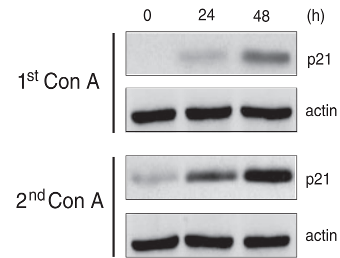

FIGURE 3. p21 expression in wt T cells after Con A treatment. p21 protein levels in T cells were measured in Western blot at the indicated times after primary or secondary Con A stimulation. Time point 0 h for the second Con A stimulation corresponds to the end of IL-2-dependent expansion.

To examine directly whether lack of p21 affected the apoptosis rate, we measured activation-induced cell death (AICD) by TUNEL after secondary Con A stimulation and found no difference between $p21^{-/-}$ and wt T cell apoptosis levels (Fig. 2*A*). To confirm that AICD and p21 deficiency are unrelated, we treated IL-2-expanded T cells with anti-CD3 or FasL. PI staining showed no difference in apoptosis induction between $p21^{-/-}$ and wt T cells at 12 h posttreatment (Fig. 2*B*).

We also measured apoptosis and cell cycle by PI staining after secondary challenge, and found no differences in apoptosis induction 24 or 48 h after secondary Con A challenge (Fig. 2*C*, *top*). As anticipated, we detected a larger proportion of cycling $p21^{-/-}$ than of cycling wt T cells (Fig. 2*C*, *top*). To examine T cell proliferation independently of apoptosis, T cells received a secondary Con A challenge in the presence of the apoptosis inhibitor zVAD that inhibits apoptosis via caspase inactivation (33). zVAD-treated $p21^{-/-}$ and wt T cells did dot die by apoptosis, but both showed a transient increase in proliferation at 24 h postchallenge, which normalized by 48 h (Fig. 2*C*, *bottom*). The proportional increase in $p21^{-/-}$ T cell proliferation compared with that of wt cells was nonetheless similar with or without zVAD at 24 and 48 h after secondary stimulation (Fig. 2*C*, compare *top* and *bottom*). The data show that apoptosis does not affect the hyperproliferative capacity of T cells conferred by p21 deletion.

p21 expression after primary and secondary Con A stimulation of wt T cells

The data showed a critical role for p21 in proliferation of activated lymphocytes after secondary challenge, but not after TCR-mediated activation of naive T cells. To clarify the differential p21 requirement in these two cases, we examined p21 protein expression kinetics during stimulation of naive and activated wt T cells. p21 levels were undetectable by Western blot in steady-state naive T cells (Fig. 3). At 24 h poststimulation, p21 expression was evident, and rose to higher levels by 48 h (Fig. 3); $p21^{-/-}$ and wt T cells nonetheless proliferated similarly, as tested at 72 h poststimulation (Fig. 1*A*, *left*). Although IL-2-expanded T lymphocytes had low p21 levels, p21 expression rose rapidly at 24 h after secondary Con A challenge and continued to rise at 48 h (Fig. 3), with overall levels higher than after primary Con A stimulation.

Consecutive TCR stimulations without IL-2 expansion require p21 for T cell proliferation control

After IL-2-dependent stimulation, a secondary TCR challenge led to excessive cycling of apoptosis-surviving $p21^{-/-}$ T cells. To

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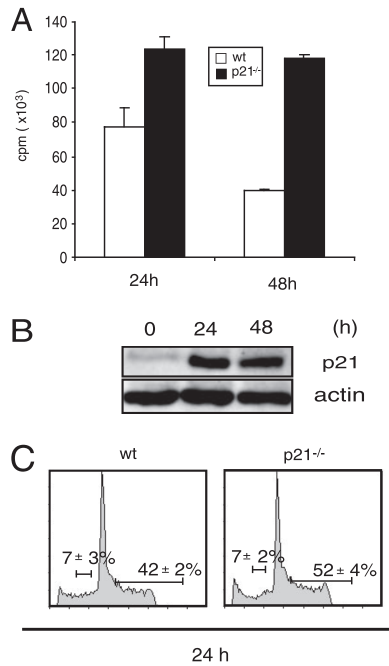

FIGURE 4. Proliferative responses of CD4$^+$ T cells after two consecutive Con A treatments. CD4$^+$ T splenocytes were subjected to two consecutive Con A challenges without intermediate IL-2 treatment. *A*, [^{3}H]Thymidine incorporation 24 and 48 h after the second Con A treatment. Values represent mean ± SD ($n = 5$) for p21$^{-/-}$ and wt T cells (24 h, $p < 0.0008$; 48 h, $p < 0.0001$). *B*, p21 expression in T cells was measured in Western blot at the indicated times after the second Con A stimulation (the 0-h time point corresponds to the end of the first Con A stimulation). *C*, PI staining of T cells 24 h after the second Con A treatment. The proportion of apoptotic and cycling T cells is represented as mean ± SD ($n = 5$).

determine whether IL-2 expansion was required for p21 deficiency-dependent T cell hyperproliferation after secondary stimulation, we subjected T cells to two successive TCR challenges without intermediate IL-2 treatment. CD4$^+$ cells were washed 72 h after primary stimulation and immediately restimulated with Con A. This type of stimulation revealed a proliferative advantage of p21$^{-/-}$ compared with wt T cells 24 and 48 h after secondary challenge (Fig. 4*A*). Under these conditions, however, p21$^{-/-}$ or wt T cells did not undergo apoptosis (Fig. 4*C*). The results show that the p21 requirement in T cell cycle regulation is manifested during the second round of TCR stimulation and is independent of the IL-2 expansion stage.

We next examined p21 protein levels in p21$^{+/+}$ T cells before and after secondary challenge. The data showed a considerable increase in p21 protein after secondary Con A stimulation compared with primary stimulation (Fig. 4*B*), suggesting that p21 levels are critical for control of repetitive T cell proliferation.

We also analyzed the cell cycle profile of p21$^{-/-}$ and wt T cells after secondary Con A stimulation. Apoptosis was minimal for both p21$^{-/-}$ and wt T cells under these stimulation conditions (Fig. 4*C*), whereas, as anticipated, we detected a large proportion of cycling p21$^{-/-}$ compared with wt T cells. These results corroborate the data showing that p21$^{-/-}$ T cell hyperproliferation is apoptosis independent.

Activation/memory phenotypes of T cells after repeated stimulation

T cells were considered to be of memory phenotype when they expressed CD44^{high} and CD62L^{low}. At the end of the first Con A stimulation were no proliferative differences between p21$^{-/-}$ and wt T cells detected (Fig. 1*A*, *left*); there was no substantial shift to memory phenotype (Fig. 5*A*). At the end of the IL-2 expansion, the proportion of memory T cells was increased (Fig. 5*B*). Hyperproliferation of p21$^{-/-}$ T cells during this treatment suggests that p21 control of proliferation is associated with a specific activated/memory phenotype. This is supported by the increased representation of memory CD62^{low} T cells within the population of CD4$^+$CD44$^+$-activated T cells in p21$^{-/-}$ vs wt cultures ($p < 0.0005$) (Fig. 5*B*). Furthermore, memory T cells generated 24 h after secondary Con A challenge (Fig. 5*C*) hyperproliferated in the absence of p21 at 48 and 72 h postchallenge (Fig. 1*D*).

After two successive Con A stimulations with no intermediate IL-2 culture, there was a marked shift toward memory phenotype 24 h after secondary Con A stimulation (Fig. 5*D*), again associating p21 deficiency with increased activated/memory T cell proliferation (Fig. 4*A*).

Memory T cell accumulation and autoimmune disease in p21$^{-/-}$ mice

The association of p21 deficiency with increased activated/memory T cell proliferation has in vivo implications. Representation of memory T cells (CD4$^+$CD44^{high}CD62L^{low}) within the CD4$^+$ T cell population in the spleens of 2-mo-old C56BL/6 p21$^{-/-}$ and wt mice was similar (<3%). However, we observed enhanced accumulation of memory T cells in spleens of 6-mo-old p21$^{-/-}$ female and male mice compared with wt controls ($p < 0.0001$ for females and $p < 0.008$ for males; Fig. 6*A*). Memory T cell accumulation was significantly higher in p21$^{-/-}$ female mice compared with

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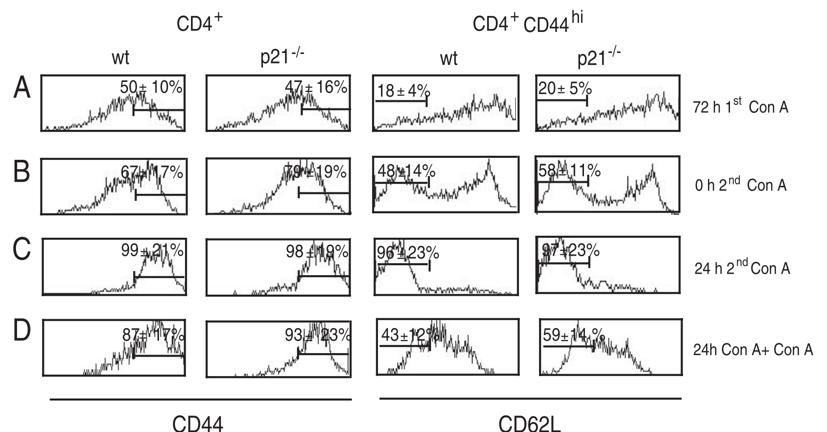

FIGURE 5. Phenotype characterization of CD4$^+$ T cells after in vitro TCR challenge. T cells were triple stained for CD4, CD44, and CD62L. Levels of CD44 expression in CD4$^+$ cells or of CD62L expression in CD4$^+$CD44^{high} cells are shown as follows: at the end of primary Con A stimulation (*A*), at the end of IL-2-dependent expansion (*B*), 24 h after the secondary Con A stimulation that follows IL-2 expansion (*C*), and 24 h after the secondary Con A stimulation without intermediate IL-2 expansion (*D*). Data are represented as mean ± SD ($n = 4$).

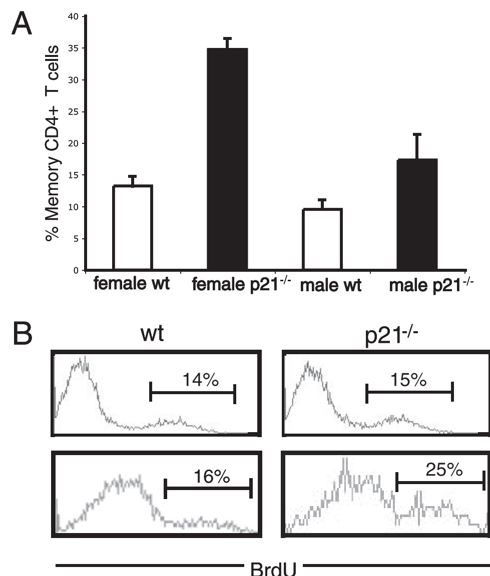

FIGURE 6. Accumulation of memory phenotype $CD4^+$ T cells in $p21^{-/-}$ mice. *A*, Proportion of memory $CD4^+CD44^{high}CD62L^{low}$ T cells in 6-mo-old mouse spleens. *B*, In vivo BrdU incorporation by $CD4^+$ $CD44^{high}$ T splenocytes from 2-mo (*top*)- and 6-mo (*bottom*)-old mice given BrdU containing water. Data are shown from representative mice; values are mean ± SD ($n = 6$; $p < 0.0015$).

male counterparts ($p < 0.005$; Fig. 6*A*). Comparable increases in memory $CD4^+$ T cell accumulation in female vs male mice were reported for mixed background (129/Sv × C57BL/6) $p21^{-/-}$ mice (4). $CD8^+$ T cells did not accumulate preferentially in $p21^{-/-}$ mixed background mice (4). Similarly, $CD8^+$ memory T cells were equally represented in 6-mo-old $p21^{-/-}$ C57BL/6 (13 ± 4%) and wt controls (12 ± 3%). Overall, the data show that in vivo accumulation, and therefore homeostasis, of memory $CD4^+$ T cells depends on p21.

We next used BrdU incorporation to test the effect of p21 deficiency on in vivo memory T cell proliferation. Although $CD4^+$ T cells from 2-mo-old $p21^{-/-}$ and wt mice proliferated similarly (Fig. 6*B*, *top*), we found that $CD4^+$ memory T cells from 6-mo-old $p21^{-/-}$ mice had an in vivo proliferative advantage compared with controls (Fig. 6*B*, *bottom*).

We previously reported that 129/Sv × C57BL/6 $p21^{-/-}$ mice, particularly females, develop autoimmune characteristics, including splenomegaly, lymphadenopathy, and severe lupus-like disease (4). To exclude genetic background interference, in this study we analyzed lupus autoimmunity development in C57BL/6 $p21^{-/-}$ mice. At 12 mo of age, C57BL/6 $p21^{-/-}$ mice had normal spleens and LNs, but developed high levels of anti-dsDNA Ab of all IgG isotypes compared with age-matched C57BL/6 controls (Fig. 7*A*). Autoantibody production by C57BL/6 $p21^{-/-}$ mice was nonetheless much lower than in mixed background counterparts (Fig. 7*B*). By 12 mo, male and female mice had glomerular IgG deposits (Fig. 7*C*) and developed moderate glomerulonephritis (Fig. 7*D*; scores: $p21^{-/-}$ females, 2.4 ± 0.6; $p21^{-/-}$ males, 2.2 ± 0.4 vs wt females and males, 0.0 ± 0.0; $n = 10$ mice/group). Because glomerulonephritis did not reach acute scores, C57BL/6 $p21^{-/-}$ mouse survival was high at 1 year of age (data not shown). Lupus-like disease was less severe in C57BL/6 $p21^{-/-}$ mice than in mixed background $p21^{-/-}$ females (4), which developed lethal glomerulonephritis at 9–12 mo. These data show that full-blown lupus development requires p21 deletion as well as elements from the 129/Sv × C57BL/6 genetic background.

p21 deficiency enhances T cell memory responses in $p21^{-/-}$ mice

The results indicated that $p21^{-/-}$ T cells hyperproliferate and that p21 acquires a role in cycling regulation in activated/memory cells. We tested whether the hyperproliferation seen in in vitro generated $p21^{-/-}$ activated/memory T cells could also be observed in vivo. Sorted memory ($CD4^+CD44^{high}CD62L^{low}$) and naive ($CD4^+$ $CD44^{high}CD62L^{high}$) T cells from 6-mo-old $p21^{-/-}$ and wt mice

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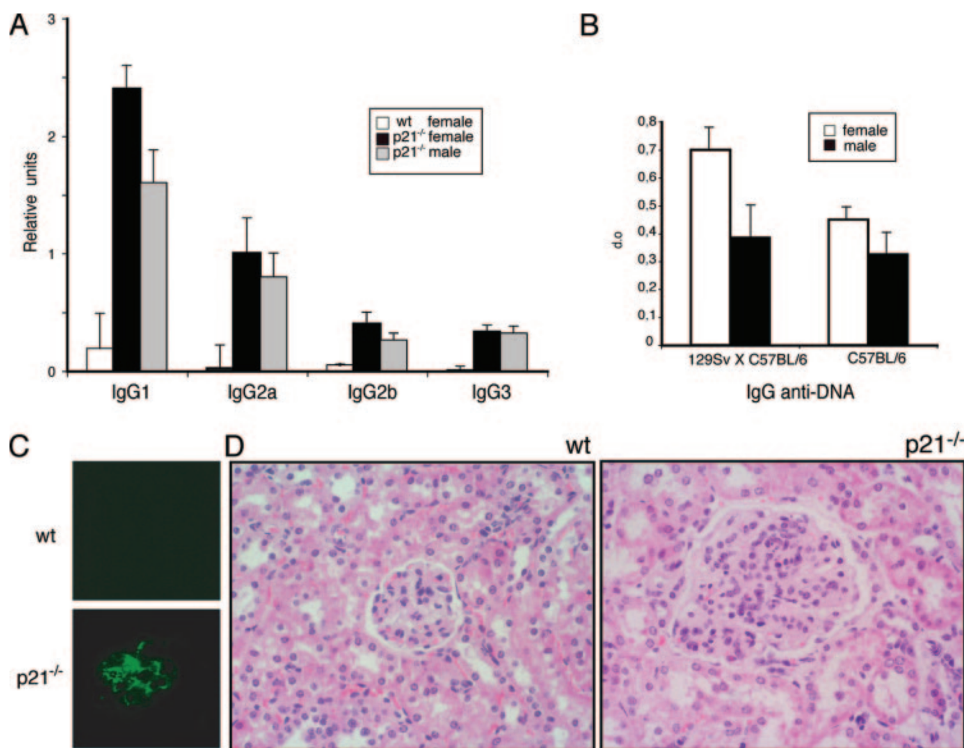

FIGURE 7. Lupus-like disease in C57BL/6 $p21^{-/-}$ mice. *A*, Serum concentrations of anti-dsDNA IgG isotypes in 12-mo-old wt ($n = 6$) and $p21^{-/-}$ female ($n = 6$) and male mice ($n = 6$). *B*, Serum concentrations of total anti-dsDNA Ab from 9-mo-old 129/Sv × C57BL/6 $p21^{-/-}$ and C57BL/6 $p21^{-/-}$ mice ($n = 5$). *C*, Characteristic IgG deposits in glomeruli of 12-mo-old C57BL/6 wt and $p21^{-/-}$ female mice. *D*, Glomeruli of 12-mo-old mice. The glomerulus of the $p21^{-/-}$ mouse shows glomerulonephritis (3^+) with hypertrophia and hyperplasia of mesangial cells, as well as expanded mesangium that obliterated the capillary turf. Original magnification ×40. For *A* and *B*, values are mean ± SD.

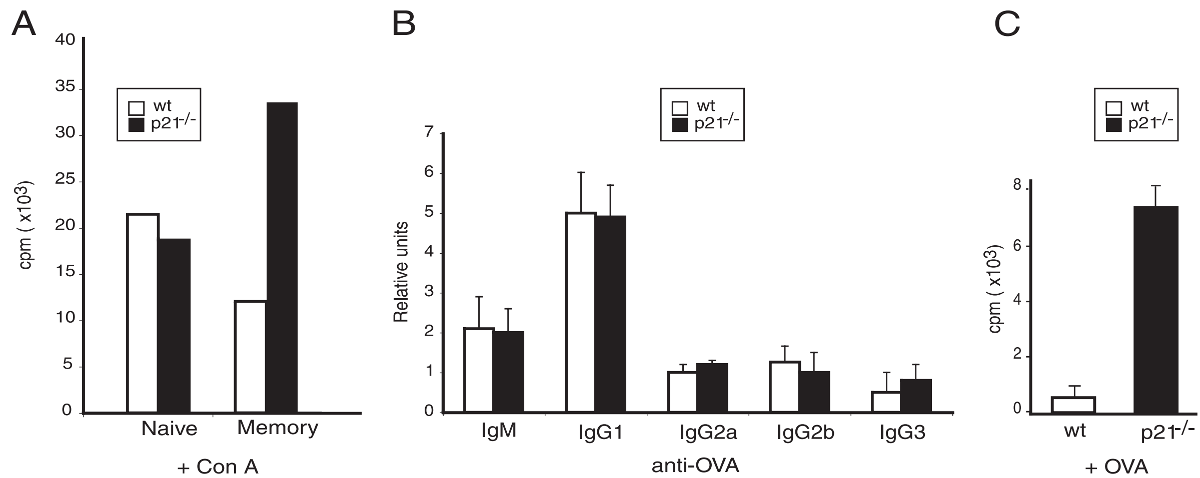

FIGURE 8. Effect of p21 deficiency on in vivo generated memory T cells. *A*, [^{3}H]Thymidine incorporation rates of sorted CD4$^+$CD44^{high}CD62L^{low} T cells from 6-mo-old wt and p21$^{-/-}$ mice; cells were Con A and IL-2 stimulated in vitro for 48 h. Data correspond to pooled cells from four mice in one of three independent experiments. *B*, Anti-OVA IgG isotype levels in serum of wt (n = 8) and p21$^{-/-}$ female mice (n = 8) 1 wk after the OVA plus IFA boost. *C*, Bead-purified CD4$^+$ cells were isolated 7 days after the OVA plus IFA boost. The wt and p21$^{-/-}$ cells were cultured with OVA and irradiated splenocytes, and proliferation was measured 24 h postactivation by [^{3}H]thymidine incorporation. Values represent mean ± SD (n = 4) of one of two experiments ($p < 0.001$).

were Con A stimulated. As predicted, p21$^{-/-}$ memory T cells proliferated at much higher rates than did controls by an average of 42 ± 8% (n = 3; $p < 0.003$) (Fig. 8*A*). p21 deletion did not influence naive T cell proliferation (Fig. 8*A*). These data suggest a role for p21 in memory T cell proliferation and homeostasis in vivo.

To determine whether p21 deficiency affected the T memory response to a model Ag in mice, we immunized p21$^{-/-}$ and wt mice with OVA in CFA. OVA challenge in IFA 3 wk after primary immunization produced no difference in anti-OVA Ab levels in the two mouse groups (Fig. 8*B*). Independently of the anti-OVA Ab response, we tested whether p21 deficiency affected T cell memory responses after OVA immunization. Purified CD4$^+$ splenocytes from p21$^{-/-}$ and wt mice were obtained 7 days after a second OVA boost, and were cultured with OVA and irradiated splenocytes. p21$^{-/-}$, but not wt T cells showed an immediate response to OVA at 24 h postactivation (Fig. 8*C*). These results corroborate in vitro findings that link p21 deficiency to a major increase in memory T cell proliferation.

Discussion

Although previous studies showed that p21 has a role in various T cell proliferation systems, the conditions that render T cell proliferation p21 dependent remained undefined (4, 8, 14, 28, 29). In this study, we explored the role of p21 in T cell proliferation of activated/memory T cells and reached the following conclusions: 1) p21 does not affect naive T cell proliferation, but exerts its control on activated/memory T cells; 2) p21 does not affect AICD, but regulates proliferation of memory T cells that survive AICD; 3) p21 regulates responses of in vivo generated memory T cells; and 4) p21 deficiency increases CD4$^+$ memory cell accumulation in aging C57BL/6 p21$^{-/-}$ mice in a sex-dependent manner and leads to typical lupus glomerulonephritis.

Because one goal was to dissect the exact role of p21 on the immune response independently of mixed background influences, we analyzed C57BL/6 p21$^{-/-}$ mice. Memory T cells accumulate in C57BL/6 p21$^{-/-}$ mice in vivo, more so in females than in males, suggesting an effect of the female hormonal environment in association with p21 deletion. This sex difference was not translated into more acute lupus disease in female than in male p21$^{-/-}$ mice, as was the case for mixed background p21$^{-/-}$ mice (4).

C57BL/6 p21$^{-/-}$ mice develop all lupus characteristics, including moderate glomerulonephritis. The genetic elements of the mixed background (6) and the female hormonal environment that enhance lupus disease (34, 35), in conjunction with the lack of p21, promote acute disease in 129/Sv × C57BL/6 female mice (4, 8). We thus conclude that for full-blown disease development in females, both p21 deletion and other genetic components are necessary. This result was anticipated (4, 7) due to the multifactorial nature of lupus. Indeed, genetic background is a determining factor for lupus disease development in mice with mutations for lupus-related genes (36, 37); thus, whereas lack of Fas leads to full-blown lupus in combination with the MRL background, it causes milder disease in C57BL/6 mice (38).

To examine whether p21 controls memory T cell proliferation, we used a classical model of memory T cell derivation, in which homeostasis is presumed to occur by apoptosis (39). After Con A stimulation and IL-2 expansion, T cells are rechallenged with Con A. Although the majority of the cells die by Fas-mediated apoptosis, a small proportion of memory phenotype T cells survives. Using various approaches, we concluded that p21 does not affect AICD in the C57BL/6 background.

In a previous study, it was reported that, in the BXSB atypical lupus model, TCR-stimulated BXSB p21$^{-/-}$ T cells hyperproliferate and exhibit similar apoptosis levels compared with BXSB controls 24 h after stimulation (10, 13). Nevertheless, increased apoptosis was detected 3 days after secondary culture with anti-CD3 Abs for BXSB p21$^{-/-}$. We have shown in this study by a variety of different systems that p21 deficiency does not influence AICD. However, p21$^{-/-}$ T cells that survive apoptosis 24 h after secondary challenge proliferate excessively compared with controls and reach the highest degree of hyperproliferation 3 days after secondary challenge. These findings raise the possibility that the increased apoptosis observed in BXSB mice 3 days after secondary challenge may be an indirect effect of the hyperproliferation of T cells, an issue that could perhaps be clarified in a future study. On the basis of the reduced autoimmune manifestations exhibited

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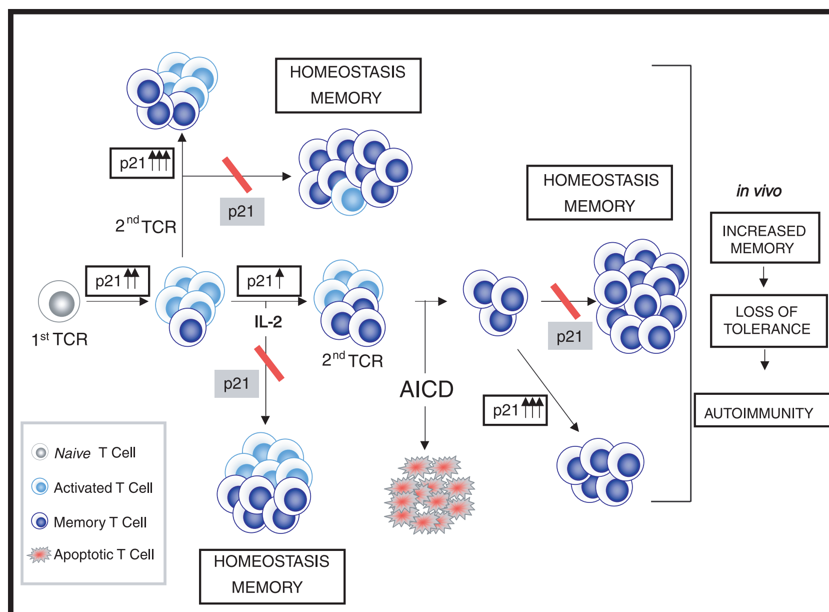

FIGURE 9. A model showing p21 control of activated/memory T cell proliferation. The p21 control points are represented by red bars, and indicate that p21 deficiency leads to hyperproliferation of activated/memory T cells. This hyperproliferation can be translated into deregulated homeostasis of memory T cells. p21 expression levels at different stages of T cell activation are depicted by a number of arrows ranging from 1 to 3.

by male BXSB p21$^{-/-}$ mice, it was concluded that increased apoptosis of memory male BXSB p21$^{-/-}$ T cells led to their reduced representation and disease improvement (13). This view, however, has been challenged by others (14) because p21$^{-/-}$ T cells from BXSB female mice (10) presumably exhibit increased apoptosis like their male counterparts (13), but memory T cells are not reduced in female mice. It appears that the disease improvement in male p21$^{-/-}$ BXSB mice may represent an interaction between the p21 and the *Yaa* gene, in particular at the B cell level, because it has been undoubtedly shown that the direct effect of the *Yaa* gene occurs rather at the B, than at the T cell level (40, 41). Finally, the particular BXSB background may confound the effects of p21 in memory T cell generation and autoimmunity, something that is now evident from our studies with C57BL/6 p21$^{-/-}$ mice. We believe that the nonautoimmune C57BL/6 background offers more clear-cut conditions for defining the role of p21 in T cell apoptosis and/or proliferation.

Our data from the repeated T cell activation experiments showed that p21 does not affect AICD. Apoptosis-resistant T cells require p21 to control their proliferation, however, which may comprise a homeostatic mechanism that regulates memory T cell expansion. In addition to repetitive Ag stimulation, memory T cells can be derived after a single TCR stimulation (42, 43). According to this model and as shown by our data, memory T cells are generated after extensive IL-2 treatment following primary stimulation (Fig. 5*B*). In another approach to generate memory T cells, we subjected cells to two consecutive Con A stimulations. Hyperproliferation of activated/memory p21$^{-/-}$ T cells derived by these two methods confirmed that memory T cells require p21 for control of proliferation. Because apoptosis is minimal during IL-2-dependent T cell expansion and after two consecutive Con A treatments, proliferation control by p21 appears to be the major pathway limiting activated/memory T cell expansion. These results suggest that certain forms of memory T cell homeostasis can be apoptosis independent and are regulated by cell cycle control mechanisms. Our model (Fig. 9) shows the experimental approaches used to generate activated/memory T cells and indicates p21 regulation points of T cell proliferation, as well as immune functions that may be controlled by p21, such as memory and homeostasis.

Our data showing that deletion of p21 affects the proliferation of activated/memory T cells, but not that of naive T cells after TCR challenge may explain an apparent conflict among previous studies. Although primary stimulation of naive T cells was shown to be p21 independent by different groups (4, 8, 27), another study argued that p21 deficiency led to increased proliferation after primary activation (7). These authors of the latter study used T cells from LN of older (4- to 6-mo-old) mice that presumably include a proportion of activated/memory T cells. Therefore, the increased proliferation possibly represented the response of activated/memory LN cells and not of naive T cells to TCR challenge. We believe that as we examined primary proliferation at different time points, under varying strength stimulation conditions, and using naive cells from older and younger mice, it is established that p21 deficiency does not affect the proliferation of naive TCR-challenged T cells.

We show a role for p21 in the regulation of activated/memory T cell proliferation, although the precise mechanism by which p21 exerts its control is not defined. A possible explanation for p21 control of T cell proliferation is the degree of p21 expression, represented schematically in Fig. 9 in association with the different types of stimulation. p21 expression levels in wt T cells are higher after secondary than after primary stimulation (Fig. 9). Because p21 does not influence proliferation after primary activation, it could be suggested that the degree of p21 expression is critical for T cell proliferation control. Alternatively, despite the low p21 levels expressed by T cells during IL-2 treatment (Fig. 9), p21 has a critical role in regulating proliferation during IL-2 expansion. This apparent paradox could be explained by considering that T cell differentiation status may potentiate the ability of p21 to regulate proliferation. Although the mechanism for p21 regulation of T cell proliferation must be addressed in detail, our data point to the possibility that both the T cell differentiation stage and p21 expression levels may influence this mechanism.

One question raised by our results is why there is a requirement for p21 in memory T cell proliferation and not in naive T cells. Apparently, the cell cycle machinery is differentially regulated in the two different types of T cells. It could be proposed that an increase in p21 may lead to a form of replicative senescence in memory phenotype cells because replicative senescence is induced

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after extensive T cell cycling (44). This possibility, however, seems unlikely, because, in experiments of only two constitutive Con A treatments in which senescence is unlikely to play a role, $p21^{-/-}$ T cells proliferated more than controls (Fig. 4*A*). What seems more likely is that primary stimulation of T cells leads to a memory phenotype that potentiates the requirement for p21 in the control of proliferation. An explanation for the differential role of p21 in naive and memory T cell proliferation could be based on epigenetic modifications that occur during the transit of naive to memory T cells (45). Thus, epistatically modified memory T cells are programmed to respond rapidly after Ag encounter (45), and therefore, may require p21 to subside this excessive response.

The association between p21 deficiency and T cell memory has in vivo implications, because immunization studies with OVA showed that p21 deficiency confers hyperresponsiveness on OVA-specific T cells. The fact that the exacerbated T cell response is not translated to increased Ab production might reflect B cell response inhibition by lack of p21. Indeed, in caspase-3 knockout mice, which have an overactive B cell response, p21 deletion reduces B cell hyperproliferation (46).

The hyperresponsiveness of $p21^{-/-}$ T cells after immunization suggests that in vivo generated memory T cells require p21 to control their expansion. This was confirmed because memory T cells from $p21^{-/-}$ mice hyperproliferated after TCR challenge. By regulating proliferation of memory T cells, p21 thus may control their homeostasis, assuring an adequate proportion of memory cells in the total T cell population. To explain anti-DNA Ab production and autoimmunity in $p21^{-/-}$ mice, we suggest that p21 deficiency may lead to memory T cell hyperproliferation and, following persistent encounter with autoantigens, to deregulated homeostasis and loss of tolerance.

We examined the role of p21 in the control of activated/memory T cell proliferation in vitro and the association of this property with memory cell responses. p21 control of activated/memory T cell proliferation in a variety of conditions assigns this cell cycle inhibitor the role of a broad controller of memory T cell proliferative responses. Our findings also link p21 with in vivo memory T cell proliferation, and establish grounds for further investigation of the role of p21 in memory T cell responses and homeostasis, and in tolerance establishment.

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Disclosures

The authors have no financial conflict of interest.

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