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Deletion of p21 (WAF-1/Cip1) Does Not Induce Systemic Autoimmunity in Female BXSB Mice

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Cell cycle, apoptosis, and replicative senescence are all influenced by the cyclin-dependent kinase inhibitor, p21. It was previously reported that deletion of p21 in 129/Sv × C57BL/6 mixed genetic background mice induced a severe lupus-like disease, almost exclusively in females. However, we did not confirm this finding in an independently derived stock of 129/Sv × C57BL/6 p21−/− mice. To further address this discrepancy, we examined the effects of p21 deletion in BXSB female mice that develop late-life, mild lupus-like disease. Survival, polyclonal Ig, anti-chromatin Abs, and kidney histopathology in these mice were unremarkable and identical to wild-type littermates for up to 14 mo of age. We conclude that p21 deficiency does not promote autoimmunity even in females of a predisposed strain. The findings indicate that the use of mixed background 129/Sv × C57BL/6 mice to study effects of gene deletions in systemic autoimmunity may be confounded by the genetic heterogeneity of this cross. We suggest that studies addressing gene deletion effects in systemic autoimmunity should use sufficiently backcrossed mice to attain genetic homogeneity, include wild-type littermate controls, and preferentially use congenic inbred strains with late-life lupus predisposition to emulate the polygenic nature of this disease. The Journal of Immunology, 2002, 168: 5928–5932.

We created congenic female p21-deficient BXSB mice to further address whether p21 has autoimmune disease-promoting activity and to clarify the above discrepancy. Male BXSB mice develop an early-life severe lupus-like disease promoted by an as yet undefined Y-chromosome-associated accelerator of autoimmunity (Yaa) (12). This accelerator is insufficient in itself to induce severe lupus, and many other genes within this background need to act in concert for full disease expression (12). Female BXSB mice carry these ancillary disease-promoting genes and, when crossed with New Zealand Black (NZB) or New Zealand White (NZW) males, generate F1 hybrids with a lupus-like disease resembling that of the (NZB × NZW)F1 cross (13). We found that even in this lupus-predisposed background there is no significant difference in disease parameters between the female p21+/− and p21−/− littermates, including autoantibodies, glomerulonephritis (GN), and mortality. These findings unequivocally show that p21 deficiency does not promote the induction of systemic autoimmunity.

Materials and Methods

Mice

129/Sv × C57BL/6 p21−/− mice, a gift from P. Leder (Harvard Medical School, Boston, MA) were backcrossed to the BXSB strain followed by final intercrossing of heterozygous BXSB p21+/− offspring. Homozygously deleted p21−/− female BXSB mice from generations 8–11 were compared with control female and male p21+/− littermates. Genotyping for the p21 deletion was performed by PCR, as described (14). Mice were maintained under specific pathogen-free conditions, and procedures were performed according to the guidelines of the Institutional Animal Research Committee of The Scripps Research Institute (La Jolla, CA).

Serologic analysis

IgG and autoantibody levels were measured by ELISA (15). Briefly, serial dilutions of mouse sera were captured on 96-well plates coated with either goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) or mouse chromatin (3–5 μg/ml), purified as described (16). Bound IgG subclasses were detected using alkaline phosphatase-conjugated IgG subclass-specific Abs (Caltag Laboratories, Burlingame, CA). Standard curves for each subclass were generated using calibrated mouse serum (Accurate Chemical and Scientific, Westbury, NJ).
Flow cytometry

Splenocyte T and B cell subsets were quadruple-stained with combinations of Abs to CD4, CD8, CD19, CD21, CD23, CD25, CD27, CD44, and CD45R/B220. PBMC were stained with anti-CD11b (Mac-1). Samples were acquired on a FACSCalibur and analyzed with CellQuest analysis software (both from BD Biosciences, Mountain View, CA). All Abs were purchased from BD PharMingen (La Jolla, CA).

Kidney disease

p21-deleted and control littersmates were followed for survival up to 14 mo of age. AzoSTIX strips (Bayer, Elkhart, IN) were used to measure blood urea nitrogen (BUN) and graded on a 1–4 scale (5–90 mg/dl). Histologic examination of periodic acid Schiff-stained kidneys was done in a blind manner, and severity of GN was defined on a scale of 0–4+ (0 = no pathology, 1 = minimal mesangial thickening, 2 = noticeable increases in both mesangium and glomerular cellularity, 3 = the preceding features plus inflammatory exudates and/or capsular adhesions, and 4 = obliteration of glomerular architecture involving ≥70% of glomeruli) (17). Kidney sections were embedded in OCT, fixed in ice-cold acetone, and blocked with 10% horse serum in PBS. Sections were then incubated with anti-IgG-FITC (Vector Laboratories, Burlingame, CA) and scored by comparing glomerular FITC intensity after equal exposure times. A 5-mo-old male BXSB mouse was used as a reference score of 4.

In vitro proliferation assays

Splenocytes from p21+/− and p21−/− 14 mo-old BXSB female mice (n = 5 per group) were seeded in triplicate (1 x 10^6 cells/well) with soluble anti-CD3 (5 μg/ml) (BD PharMingen) into 96-well plates coated with increasing concentrations of anti-CD3 and incubated for 48 h in serum-free HL-1 medium. Thereafter,[^H]Thymidine (1 μCi) was added and incorporation measured 15 h later. Subsequently, kinetics of proliferation was assessed by plating pooled splenocytes (5 x 10^6 cells/well) on optimally anti-CD3 (10 μg/ml) coated 96-well plates plus soluble anti-CD28 (5 μg/ml; n = 5 mice per group) and measuring[^H]Thymidine incorporation every 24 h for 6 days (18).

Apoptosis induction

Splenocytes from 14-mo-old female p21+/+ and p21−/− BXSB mice were plated (10^6 cells/ml) for 48 h with soluble anti-CD3 (0.5 μg/ml) and harvested, and viable cells were purified over Ficoll-Histopaque (Sigma-Aldrich, St. Louis, MO), washed, and replated (4 x 10^5 cells/well) into 96-well plates with plate-bound anti-CD3 (BD PharMingen) (19). At 24-h time points, cells were stained with either anti-CD4 or anti-CD8 plus annexin V and propidium iodide (to exclude dead cells), and the percentage of apoptotic cells (annexin V+propidium iodide−) in each T cell subset was determined by FACS.

Statistics

The Student t test was used for group mean comparisons and survival was analyzed by the Kaplan-Meier method with comparisons by log-rank test. Values of p < 0.05 were considered significant.

Results

Survival of female BXSB p21+/+ and p21−/− mice

To assess the possible impact of p21 on the development of a lupus-like disease in a spontaneous model, female BXSB mice deficient for p21 were created and the severity of autoimmune parameters was compared with p21+/− littersmates. Female BXSB mice possess all the necessary genetic components, other than the Yaa gene, for lupus development. Therefore, if p21 has the capability to induce autoimmunity in normal genetic background female mice, its effect should be greatly magnified in a predisposed mouse with mild autoimmune manifestations. No deaths were observed in females of either group up to 14 mo of age. In contrast, congenic p21+/+ BXSB male littersmates showed 50% mortality at 5.6 mo and 100% mortality by 6.3 mo. These data regarding the male p21+/+ BXSB littersmates indicate that the female congenic line contains all the major BXSB lupus susceptibility genes except, obviously, the Yaa.

Serum analysis in female BXSB p21−/− mice

Serum polyclonal IgG levels were measured in p21−/− and p21+/− female littersmates at 14 mo of age. Both the wild-type and the p21-deleted mice exhibited slight but equal increases in levels of IgG compared with a non-predisposed mouse (i.e., C57BL/6) (Fig. 1, upper panel). Nevertheless, total IgG levels from both female genotypes were ~3- to 4-fold lower than 4-mo-old wild-type male littersmates (~8 vs ~25 mg/ml). Similarly, anti-chromatin titers at 14 mo of age showed no significant differences between the two female groups with low and equivalent concentrations of the IgG2a and IgG2b subclasses detected (Fig. 1, lower panel). These data are consistent with previous reports of disease progression in female BXSB mice (20) and do not support accelerating effects by p21 deficiency.

Lymphoid cell analysis

Weights and cellular composition of spleen and lymph nodes (LN) were also analyzed (Table I). A slight and similar splenomegaly was observed in both female genotypes (approximately two times the weight and cellular numbers of C57BL/6 mice; p < 0.05), but there was no increase in LN weights. Similarly, CD4+ and CD8+ T cell populations were equivalent between p21−/− and p21+/− female BXSB mice and, as commonly observed in aged mice (21), a large proportion of the CD4+ T cells were of the activated/memory phenotype (CD4+CD44hi). Likewise, total B cells (CD45R/B220) as well as memory phenotype (CD27+) B cells were equivalent in the two groups (Table I).

Monocytosis in female BXSB p21−/− mice

The accumulation of an unusual Mac1+ monocyte population in the peripheral blood of young male, but not young female, BXSB mice has been reported (22). We did not observe an increase of this population in the p21+/+ and p21−/− female groups up to 8 mo of age. However, surprisingly, we found high numbers of Mac1+ cells in both female groups (31.4 vs 32.9%) at 14 mo of age. The results suggest that monocytosis is a characteristic of the BXSB background that is accelerated in the presence of the Yaa but unaffected by p21 (Table I).

Kidney disease after p21 deletion

GN and immune complex deposit scores were equivalent in 14-mo-old female p21−/− and p21+/− mice (Table II). BUN levels,
although equal between genotypes, were marginally elevated compared with normal mice. Female BXSB mice of both genotypes showed very slight glomerular damage, with only one wild-type and one p21-deleted mouse scoring above 2+ (mesangial and epithelial cell proliferation with mild deposits) at 14 mo of age. Immune complex deposits were negative to minimal in all female mice.

In vitro proliferation of T cells in female BXSB p21−/− and p21+/+ mice

To identify the effects of p21 deletion on the proliferation of female BXSB T cells, splenocytes were stimulated in vitro using increasing concentrations of plate-bound anti-CD3 together with a fixed amount of soluble anti-CD28 (Fig. 2, upper panel). Compared with wild-type littersmates, female p21−/− T cells showed significantly greater proliferation at lower anti-CD3 concentrations, suggesting a lower threshold for TCR-induced activation in the absence of p21.

Additional kinetic experiments indicated that although female BXSB p21-deficient and p21+/+ T cells proliferated equally for up to 24 h (25,412 vs 32,094 cpm), after 48 and 72 h the p21-deficient T cells exhibited greater proliferation (110,943 vs 172,244 cpm) (Fig. 2, lower panel). Interestingly, the hyperproliferation seen in the p21−/− T cells declined at an accelerated rate compared with control T cells, reaching baseline levels by ~168 h poststimulation. These data further corroborate a decreased TCR activation threshold for the female BXSB p21−/− T cells.

Increased apoptosis in female BXSB p21−/− T cells

To determine whether increased proliferation leads to enhanced apoptosis in p21-deficient animals, p21+/+ and p21−/− T cells were initially stimulated with soluble anti-CD3 followed by religation with plate-bound anti-CD3 for up to 72 h (19). Thereafter, cells were analyzed at 24-h intervals for the frequency of annexin V+ (apoptotic) cells. At 24 h postreligation the percentage of annexin V+ T cells was similar between the p21+/+ and p21−/− cells. However, by 48 h p21-deficient CD4+ and CD8+ T cells showed a greater percentage of apoptosis than wild-type T cells (CD4+, 24.8 ± 4.7 vs 35.7 ± 2.8%, p < 0.05; CD8+, 34.8 ± 2.9 vs 47.2 ± 3.6%, p < 0.05) that was even more pronounced at 72 h (Fig. 3).

![FIGURE 2. Hyperproliferative response of female BXSB p21−/− T cells.](image)

![FIGURE 3. Increased annexin V positivity in vitro after TCR religation.](image)
Discussion

Gene knockout mice have been used as a powerful tool to help define the function(s) of genes and their role in a variety of disease processes, most notably in autoimmunity. Of the large number of such mice, >25 (almost all created on the mixed genetic background 129/Sv × C57BL/6 cross) encompassing genes affecting signal transduction, transcriptional factors, apoptosis, complement, and costimulatory molecules, among others, have been reported to develop humoral and histologic manifestations of systemic autoimmunity (23). At face value, these studies suggest that perturbations in several immunologic checkpoints can disturb the fine balance of self-tolerance and point out key molecules that need to be investigated in the spontaneous disease counterparts.

Among the many systemic autoimmunity-inducing gene deletions, the CDKI p21 was notable in that pathologic manifestations were reported to be severe and largely confined to females, despite similar increases in numbers and proliferation rates of T cells between the two sexes (10). However, in our studies with an independently derived stock of 129/Sv × C57BL/6 p21-deleted mice we did not corroborate the induction of severe autoimmunity (11). Thus, our wild-type and p21−/− mice had very similar survival rates, differing only slightly in their humoral and histologic characteristics, and there was no difference between male and female p21-deficient mice. Therefore, we concluded that p21 deficiency in itself is insufficient to induce a lupus-like disease. Nevertheless, the conflicting findings could be attributed to differences in genetic compositions of the mixed background mice analyzed and/or environmental influences.

To conclusively resolve the disparity between these two studies, we conducted the present studies with p21-deficient female BXSB mice. If p21 deletion had autoimmunity-promoting effects, these mice would constitute an optimum genetic background to validate such an outcome. Female BXSB mice contain all the necessary genetic elements for lupus development except the Yaa, which in male BXSB mice accelerates the disease (12). The autoimmune potential of female BXSB mice has further been documented by crosses with NZB or NZW mice that resulted in the development of severe lupus-like disease (13). The present study unequivocally demonstrates that, even in the disease-prone female BXSB mouse, p21 deletion cannot bring about the expression of clinically apparent disease.

A 100% survival rate was observed for the wild-type and p21−/− female BXSB littermates up to 14 mo of age, the latest point of observation, a rate in agreement with previous accounts (20). Despite the p21 deletion and increased proliferation of T cells, spleen size was only marginally increased and there was no lymphadenopathy. We hypothesized that the absence of lymphoid hyperplasia in the presence of increased T cell proliferation may be due to enhanced apoptosis. Other studies have shown that T cell proliferation and apoptosis are intimately linked (24). Indeed, increased in vitro apoptosis of the p21−/− T cells after anti-CD3 repletion was observed. This effect was also detected in p21-deficient T cells from male BXSB mice wherein a reduction in overall numbers of CD4+CD44high T cells concomitant to increased apoptosis was noted.4 Other cellular characteristics, including B cells numbers and phenotypes, and follicular and marginal zone B cells, were equal between the wild-type and p21-deficient littermate female mice. These data suggest that p21 deletion has a marked effect on T cell homeostasis but not on B cells. In addition, total Ig, anti-chromatin levels, glomerular deposition, and GN were mild in both female BXSB groups. Of note, the frequency of PBMC Mac1+ cells was increased in 14-mo-old females of both the p21+/+ and p21−/− groups. Increases in these cells, originally described in male BXSB mice (22), are highly T cell dependent (25, 26). However, the present findings indicate that this manifestation is not confined to males and that Yaa simply accelerates its development.

The combined data from our previous study with p21-deficient 129/Sv × C57BL/6 mice and those from the present study with the p21-deficient female BXSB mice make it evident that p21 deficiency is not an inducer of systemic autoimmunity. Furthermore, in a separate study we document that male BXSB mice deficient in p21 show significantly increased survival and reduced serologic, cellular, and histologic disease parameters.4

Other reports support the conclusion that the genetic heterogeneity of 129/Sv × C57BL/6 mice significantly confounds interpretations of the significance or relevance of some gene deletions to autoimmunity. For example, ~55% of C57BL/6 × 129/Ola mice deleted of the C1q gene developed signs of systemic autoimmunity, whereas 33% of the nondeleted mice also displayed some of these characteristics (27). Additionally, 129/Sv × C57BL/6 mice deleted of vitamin D binding protein reportedly showed strong defects in macrophage and neutrophil recruitment, but this effect was absent upon further backcrossing to the C57BL/6 background (28). Following further analysis, the authors concluded that the defects were not linked to the vitamin D binding protein locus but to the 129/Sv genetic background of the embryonic stem cells.

Overall, the present findings strongly indicate that studies addressing autoimmune-potentiating effects of gene deletions need to control for the effects of the 129/Sv × B6 mixed background. Detailed information must be provided pertaining to controls, which should, at the least, include wild-type littermates. Moreover, to properly evaluate and reproduce the observed effects, such studies should use fully backcrossed strains. Detailed description of the amount and specificities of autoantibodies, cellular modifications, and kidney immunohistology should also be provided so that relevance to spontaneous autoimmune disease can be adequately assessed. To increase the sensitivity of detecting genetic alterations with weaker contributions to systemic autoimmunity, strains such as BXSB females or MRL+/− mice might be used, because superimposition of single gene mutations have been shown to induce severe autoimmune disease in these strains but not in nonautoimmune strains (20). Ultimately, of course, the relevance of the various autoimmunity-developing gene deletions will have to be confirmed in humans.

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References


