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Mice Lacking the p53-Effector Gene *Gadd45a* Develop a Lupus-Like Syndrome

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Summary

This study addresses the biological function of the p53-effector genes *Gadd45a* and p21 in the immune system. We find that *Gadd45a* is a negative regulator of T cell proliferation because, compared to wild-type cells, *Gadd45a*$^{-/-}$ T cells have a lower threshold of activation and proliferate to a greater extent following primary T cell receptor stimulation. *Gadd45a*$^{-/-}$ mice develop an autoimmune disease, similar to human systemic lupus erythematosus (SLE), characterized by high titers of anti-dsDNA, anti-ssDNA, and anti-histone autoantibodies, severe hematological disorders, autoimmune glomerulonephritis, and premature death. Here we show that the lack of both *Gadd45a* and p21 dramatically accelerates the development of autoimmunity observed in each individual single-gene disruption mutant, demonstrating that these genes play nonredundant roles in the immune response.

Introduction

The Gadd45 (growth arrest and DNA damage-inducible gene) family is composed of three members: *Gadd45a* (Gadd45α/Gadd45), *Gadd45b* (Gadd45β/MyD118), and *Gadd45*g (Gadd45γ/CR6) that play pivotal roles in regulating growth arrest and apoptosis (Fan et al., 1999; Fornace et al., 1989; Vairapandi et al., 1996). *Gadd45a* is the only member of this family regulated by p53 (Kastan et al., 1992) and is a key player in important cellular processes including the maintenance of genomic stability (Hollander et al., 1999), cell growth control (Zhan et al., 1999), nucleotide excision repair (Hollander et al., 2001; Smith et al., 2000), chromatin accessibility (Carrier et al., 1999), and apoptosis (Harkin et al., 1999; Takekawa and Saito, 1998). Recently, we reported that, similar to p53-deficient mice, *Gadd45a*$^{-/-}$ mice have genomic instability exemplified by aneuploidy, chromosome aberrations, gene and centrosome amplification, and abnormalities in mitosis, cytokinesis, and growth control (Hollander et al., 1999). These findings confirmed that *Gadd45a* is one component of the p53 pathway that contributes to the maintenance of genomic stability and cell growth control. We have reported that one of the major mechanisms by which Gadd45a suppresses cell growth is by interacting with Cdc2, thereby inhibiting Cdc2/Cyclin B1 kinase activity (Zhan et al., 1999). Additionally, Gadd45a interacts with PCNA (Smith et al., 1994), p21 (Cdkn1a/Cip1/Waf1) (Zhao et al., 2000), and MEKK4/MTK1 (Takekawa and Saito, 1998), suggesting other potential mechanisms for maintaining genomic stability and/or control of cell growth. The fact that Gadd45a has been reported to activate MTK1 kinase activity, which is upstream of p38 and JNK/MAPKs, suggests that this might be a signaling pathway by which Gadd45a induces apoptosis (Takekawa and Saito, 1998).

The other two members of the Gadd45 family, Gadd45b and Gadd45g, also interact with Cdc2 (Zhao et al., 2000), p21 (Vairapandi et al., 1996; Zhao et al., 2000), PCNA (Vairapandi et al., 1996), and MEKK4/MTK1 (Takekawa and Saito, 1998) and also may have critical roles as negative regulators of proliferation. *Gadd45b* was discovered as a myeloid differentiation primary response gene activated in M1 myeloblastic leukemia cells by interleukin-6 (Abdollahi et al., 1991). *Gadd45g* was originally cloned as an IL-2 immediate early response gene (Beadling et al., 1993). These findings, taken together with the thymic hyperplasia seen in *Gadd45a* null mice, suggest that the Gadd45 family could very well have important roles in the immune system in the control of differentiation and T cell activation. Very recently, characterization of *Gadd45g*-deficient mice has demonstrated that Gadd45g is not necessary for normal mouse development and T cell proliferation (Hoffmeyer et al., 2001; Lu et al., 2001). However, *Gadd45g*$^{-/-}$ T_H1 effector cells failed to activate the p38/JNK pathways in response to TCR stimulation, resulting in abnormally low INF-γ production in these cells (Lu et al., 2001). Additionally, it has been shown that INF-γ production induced by IL-12 plus IL-18 requires Gadd45b expression (Yang et al., 2001). These findings suggest that the Gadd45 family can be critical for the function of T_H1 cells. However, whether Gadd45a or Gadd45b plays a role in the control of T cell proliferation remains unknown.

In this report, we have characterized the immune phenotype of *Gadd45a*$^{-/-}$ mice. We find that *Gadd45a* is a negative regulator of T cell proliferation induced by T cell antigen receptor (TCR)-mediated activation and that lack of *Gadd45a* leads to the development of a lupus-like syndrome. In addition, we explore the physiological roles of Gadd45a and p21 in the immune system by the characterization of mice lacking both genes.

Results

Increased Spontaneous Mortality of *Gadd45a*$^{-/-}$ and *p21*$^{-/-}$ Mice, which Is Further Increased in *Gadd45a*$^{-/-}$*p21*$^{-/-}$ Mice

Gadd45a$^{-/-}$ mice developed and bred normally and did not show signs of disease during the first 6 months of

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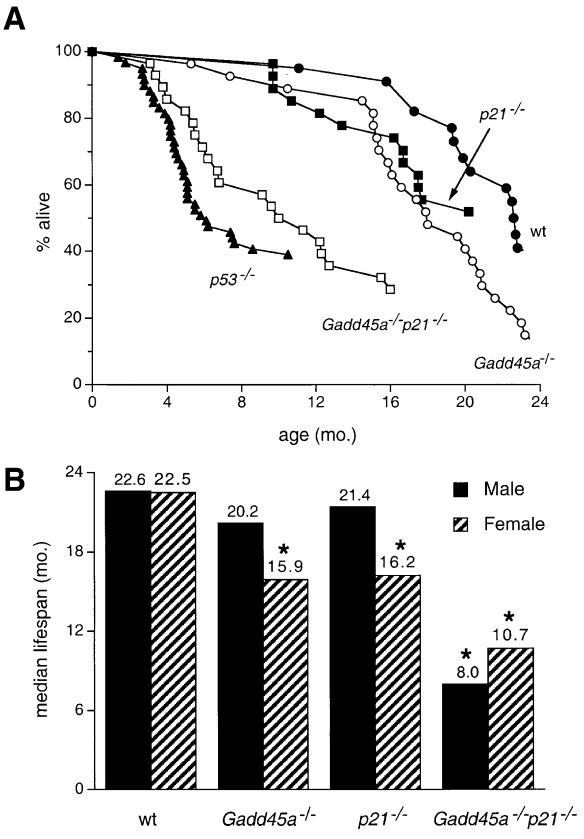

Figure 1. Median Lifespan Is Decreased in Female *Gadd45a*$^{-/-}$ and *p21*$^{-/-}$ Mice and Is Further Decreased in *Gadd45a*$^{-/-}$*p21*$^{-/-}$ Females and Males

(A) Survival of mice over time.

(B) Median lifespans.

Genotype is indicated on the *x* axis. Males, filled bars; females, striped bars. Numbers over bars indicate median lifespan in months. Animals in this study: wt, 11 F (female), 10 M (male); *Gadd45a*$^{-/-}$, 14 F, 13 M; *p21*$^{-/-}$, 12 F, 15 M; *Gadd45a*$^{-/-}$*p21*$^{-/-}$, 15 F, 13 M. Median lifespans are based on spontaneous deaths and euthanasia of moribund animals; *, $p < 0.05$.

life. However, after 7 months *Gadd45a*$^{-/-}$ mice started to die (Figure 1A). *p21*$^{-/-}$ mice had a very similar profile of spontaneous mortality compared to *Gadd45a*$^{-/-}$ mice. Moreover, when *Gadd45a*$^{-/-}$ mice were crossed with *p21*$^{-/-}$ mice to generate double null animals, there was a dramatic increase in spontaneous mortality, which began at 4 months of age. Analysis of the median lifespan by sex (Figure 1B) indicated statistically significant differences in the mortality rate of females versus males in *Gadd45a*$^{-/-}$ and *p21*$^{-/-}$ mice. *Gadd45a*$^{-/-}$ females had a statistically significant reduction in the median lifespan (16 months, $p < 0.05$) compared with wt (22 months), whereas male survival rates were similar to wt. However, *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice showed a markedly decreased median lifespan in both males (22 versus 8 months, $p < 0.05$) and females (22 versus 10 months, $p < 0.05$).

Development of Glomerulonephritis in *Gadd45a*$^{-/-}$ and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ Mice

Nine-month-old *Gadd45a*$^{-/-}$ mice and four-month-old *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice showed evidence of severe immune problems: lymphoid hyperplasia in lymph nodes and spleen and lymphocytic infiltrates in lungs, salivary glands, and chronic nephropathy. The last was evidenced by proteinuria in *Gadd45a*$^{-/-}$ females (150 ± 50 mg/dl) and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ males and females (220 ± 40 mg/dl) (wt levels were 50 ± 30 mg/dl). To explore the nature of the renal disease, histologic analysis of renal cortex was carried out in *Gadd45a*$^{-/-}$, *Gadd45a*$^{-/-}$*p21*$^{-/-}$, and wt mice at 28 and 70 weeks of age (Figure 2). Twenty-eight- and seventy-week-old wt mice had mild focal mesangial hypercellularity (Figures 2A and 2J) compared to 2-month-old wt animals. Sections from five of seven older *Gadd45a*$^{-/-}$ mice (Figure 2K) showed diffuse global mesangial proliferation involving between 40% and 100% of glomeruli. Kidney sections from 28-week-old *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice (Figure 2C) were similar to wt mice. In contrast, two of three older *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice had global mesangial and segmental endothelial proliferation involving from 10% to 100% of the glomeruli (Figure 2L). Some glomeruli contained inflammatory cells, nuclear debris, and mesangial cell interposition. There was mild dilatation of the proximal tubules and mild to severe dense perivascular mononuclear cell infiltrate. The glomerular damage in *Gadd45a*$^{-/-}$ and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice, characterized by glomerular inflammation and proliferation, suggested autoimmune glomerulonephritis. Indeed, immunofluorescent localization of murine immunoglobulins and complement in mouse kidney at 28 weeks showed increased diffuse and global granular mesangial and semilinear subendothelial deposition of IgG, IgM, IgA, and C3 in *Gadd45a*$^{-/-}$ mice compared to wt mice (Figures 2E and 2H). Deposition of immunoglobulin and C3 was further increased in *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice (Figures 2F and 2I). Therefore, mice lacking Gadd45a develop autoimmune glomerulonephropathy and the pace of immune deposition is accelerated in *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice. Although the main cause of death in *Gadd45a*$^{-/-}$ and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice appeared to be due to renal failure since no tumors were found in *Gadd45a*$^{-/-}$ mice and only four tumors were found in the 28 *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice in the study shown in Figure 1, the ages of these four mice at necropsy were 11.4, 12.2, 15.5, and 22.8 months.

Spontaneous Development of Autoantibodies in *Gadd45a*$^{-/-}$, *p21*$^{-/-}$, and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ Mice

In view of the pathological findings, we looked for other evidence of immune dysregulation. We tested whether the mice spontaneously develop autoantibodies against double-stranded DNA (anti-dsDNA), single-stranded DNA (anti-ssDNA), and/or histone. The presence of anti-dsDNA autoantibodies in the serum of *Gadd45a*$^{-/-}$, *Gadd45a*$^{-/-}$*p21*$^{-/-}$, and wt mice was assayed by the Crithidia immunofluorescence test and by enzyme-linked immunosorbent assay (ELISA). Serum from *Gadd45a*$^{-/-}$ and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice but not wild-type animals specifically recognized the nucleus and kinetoplast of Crithidia luciliae, indicating the presence of high levels of anti-dsDNA antibodies (Figures 2M–2O). To quantify the level of autoantibodies, we performed ELISA by serial serum dilutions (1:50 to 1:31250). After 7 months of age, female *Gadd45a*$^{-/-}$ developed anti-

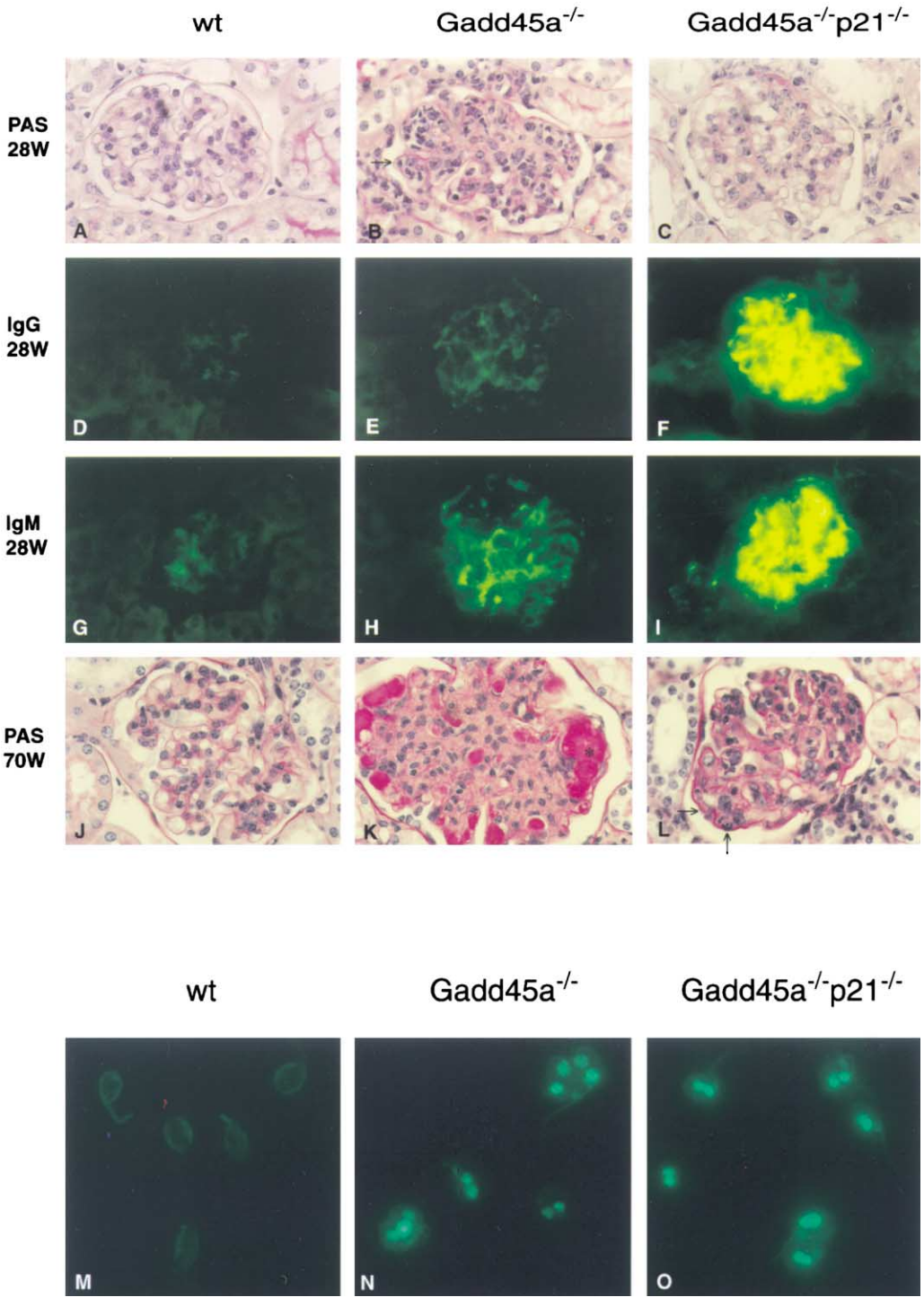

Figure 2. Renal Disease and Autoantibodies

(A–C) Glomerular disease in *Gadd45a*-deficient mice. PAS staining (A–C) of glomeruli from 28-week wt mice (A) showed no morphologic alterations in the glomeruli, which were normal in size and cellularity. Young Gadd45a$^{-/-}$ mice (B) showed increased glomerular cellularity, with mesangial cell proliferation, endothelial proliferation and swelling, inflammatory cells, and double contours, with subendothelial interposition of mesangial and inflammatory cells also present (arrow). Young *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice showed mild mesangial proliferation (C).

(D–I) Direct immunofluorescent staining of glomeruli from 28-week-old mice. Minimal IgG was detected in a focal and segmental distribution in glomeruli from wt mice (D), a small amount of mesangial IgG was present in all glomeruli from *Gadd45a*$^{-/-}$ mice (E), intense mesangial staining for IgG was present in all glomeruli from *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice (F), trace mesangial staining for IgM was present in a focal and segmental distribution in wt mice (G), mild to moderate global mesangial staining for IgM was present in *Gadd45a*$^{-/-}$ mice (H), and extensive deposition of IgM was present within the mesangium of all glomeruli of *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice (I). PAS staining of glomeruli from 70-week-old mice revealed that wt glomeruli remained indistinguishable from those of younger mice, while aged *Gadd45a*$^{-/-}$ mice showed a variable

Table 1. Spontaneous Development of Antibodies to Double-Stranded DNA, Single-Stranded DNA, and Histone in the Serum of Female *Gadd45a*$^{-/-}$ and Wild-Type Mice

	Anti-dsDNA[a]		Anti-ssDNA[b]		Anti-Histone[c]	
Genotype	wt	*Gadd45a*$^{-/-}$	wt	*Gadd45a*$^{-/-}$	wt	*Gadd45a*$^{-/-}$
Positive mice (total)	4 (n = 28)	16 (n = 27)	4 (n = 24)	14 (n = 22)	4 (n = 25)	13 (n = 26)
Average titer[d]	1:77	1:7692[e]	1:500	1:10526[e]	1:150	1:696[f]

[a] Antibodies against double-stranded DNA.
[b] Antibodies against single-stranded DNA.
[c] Antibodies against histone.
[d] Geometric mean of antibody titer of positive animals.
[e] $p < 0.0001$.
[f] $p < 0.001$.
Data is shown as number of positive females mice (9–12 months old) for autoantibodies. Statistical analysis was performed using the Wilcoxon test (p values).

bodies against dsDNA, ssDNA, and histone. At 9–12 months of age, 59% of *Gadd45a*$^{-/-}$ females were positive for anti-dsDNA while only 14% of wt were positive (Table 1). *Gadd45a*$^{-/-}$ female mice had a significant increase in the level of anti-ssDNA (64% positive) and anti-histone (50% positive) antibodies versus wt mice (16% positive). The average titer of anti-dsDNA, anti-ssDNA, and anti-histone was significantly greater in *Gadd45a*$^{-/-}$ female mice compared to wt (Table 1). Of 15 *Gadd45a*$^{-/-}$ males tested, only 20% were positive versus 13% of wt. Similar values were obtained for the level of anti-ssDNA, and no differences were noted in the level of anti-histone (13%) versus wt (13%).

Recently, Balomenos et al. (2000) reported that p21-deficient mice develop antibodies against dsDNA, ssDNA, and histone. To investigate if the additional lack of *p21* affected the spontaneous development of autoantibodies, we tested the serum of *p21*$^{-/-}$ and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice (Table 2 and data not shown). *p21*$^{-/-}$ mice had a similar profile of spontaneous development of autoantibodies as *Gadd45a*$^{-/-}$ mice. After 9–12 months, *p21*$^{-/-}$ females but not males produced high levels of anti-dsDNA (53% positive), anti-ssDNA (66% positive), and anti-histone antibodies (60% positive) compared to wt mice (13%, 13%, and 6%, respectively). Notably, *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice developed anti-dsDNA, anti-ssDNA, and anti-histone antibodies after 4 months of age. At 6–8 months of age, we noted a dramatic increase in the number of females positive for anti-dsDNA (78%), anti-ssDNA (84%), and anti-histone antibodies (70%) versus wt (12%, 16%, and 12%, respectively). Moreover, *Gadd45a*$^{-/-}$*p21*$^{-/-}$ males were also strongly positive for antibodies anti-dsDNA (73%), anti-ssDNA (77%), and anti-histone (63%) compared to wt (13%, 16%, and 8%, respectively). The average titer of anti-dsDNA, anti-ssDNA, and anti-histone in *Gadd45a*$^{-/-}$*p21*$^{-/-}$ females and males was increased compared to wt and *Gadd45a*$^{-/-}$ (Table 2). Taken together, these data show that the lack of *Gadd45a*$^{-/-}$ and *p21*$^{-/-}$ increased the prevalence of autoantibodies in female mice. In addition, the lack of both genes dramatically accelerated the development of these autoantibodies and increased their titer in both sexes.

Hematological Disorders in *Gadd45a*$^{-/-}$ and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ Mice

Some of the common findings in humans with active SLE are low numbers of leukocytes, lymphocytes, and erythrocytes in the blood. To explore these manifestations of SLE in *Gadd45a*$^{-/-}$ and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice, we carried out complete blood counts of 15 mice (7–9 months old) per genotype. Blood count samples from *Gadd45a*$^{-/-}$ and double null mice showed severe hematological disorders. Forty-seven percent of *Gadd45a*$^{-/-}$ females were leukopenic and 40% were lymphopenic, whereas only 7% of wt had low numbers of leukocytes and lymphocytes (Table 3 and Supplemental Table SA at http://www.immunity.com/cgi/content/full/16/4/499/DCI). In contrast, *Gadd45a*$^{-/-}$ males had leukocyte and lymphocyte counts similar to wt. The percentage of leukopenic and lymphopenic mice increased substantially in *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice with respect to wt and *Gadd45a*$^{-/-}$ mice at the same age. Seventy-three percent of *Gadd45a*$^{-/-}$*p21*$^{-/-}$ females and 60% of males had severe leukopenia. The mean leukocyte count of *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice was significantly lower than in wt and *Gadd45a*$^{-/-}$ mice. Moreover, 53% of *Gadd45a*$^{-/-}$*p21*$^{-/-}$ females and 46% of males were lymphopenic. In addition to the lymphopenia, *Gadd45a*$^{-/-}$ females and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice of either sex had statistically significant reductions in the total number of eosinophils and basophils (see Supplemental Table SA at http://www.immunity.com/cgi/content/full/16/4/499/DCI). These data indicate that the

increase in glomerular size and cellularity (K) more marked than in younger mice. The mesangium was expanded and contained numerous mesangial cells, and large subendothelial hyaline deposits were present and partially occluded the glomerular capillary lumens (asterisk). In aged *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice (L), there was focal global mesangial and focal endothelial proliferation. Occasional inflammatory cells were present within the glomerular tuft, and double contours were also focally present, containing mesangial cells and inflammatory cells (arrows). (M–O) The presence of anti-dsDNA autoantibodies in the serum of *Gadd45a*$^{-/-}$ and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice, detected by indirect immunofluorescence using the Crithidia luciliae test. Staining with serum from wt female mice did not show reaction against the nucleus or the kinetoplast of Crithidia luciliae. Staining with serum from *Gadd45a*$^{-/-}$ female and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ female mice showed a strong reaction against the nucleus and the kinetoplast of the Crithidia luciliae. Similar results were obtained in three independent experiments.

Table 2. Spontaneous Development of Antibodies to Double-Stranded DNA, Single-Stranded DNA, and Histone in the Serum of *Gadd45a*$^{-/-}$*p21*$^{-/-}$ and Wild-Type Mice

	Anti-dsDNA[a]		Anti-ssDNA[b]		Anti-Histone[c]	
Genotype	wt	*Gadd45a*$^{-/-}$ *p21*$^{-/-}$	wt	*Gadd45a*$^{-/-}$ *p21*$^{-/-}$	wt	*Gadd45a*$^{-/-}$ *p21*$^{-/-}$
Positive F Mice (total)	3 (n = 25)	21 (n = 27)	4 (n = 25)	25 (n = 30)	3 (n = 25)	19 (n = 27)
Average titer[d]	1:88	1:9090[e]	1:434	1:12500[e]	1:116	1:807[e]
Positive M mice (total)	3 (n = 23)	19 (n = 22)	4 (n = 24)	17 (n = 22)	2 (n = 24)	14 (n = 22)
Average titer[d]	1:88	1:8697[e]	1:150	1:11402[e]	1:50	1:907[e]

[a] Antibodies against double-stranded DNA.
[b] Antibodies against single-stranded DNA.
[c] Antibodies against histone.
[d] Geometric mean of antiobdy titer of positive animals.
[e] $p < 0.0001$.
Results are shown as number of positive females (F) and males (M) mice (7–9 months old) for autoantibodies. Statistical analysis was performed using the Wilcoxon test (p values).

lack of *Gadd45a*$^{-/-}$ increased the incidence of leukopenia and lymphopenia in female mice and that the additional loss of *p21* in the double null animals led to the development of severe leukopenia and lymphopenia in both sexes.

Gadd45a Is a Negative Regulator of T Cell Proliferation

To determine whether the lack of *Gadd45a* and *p21* affects T cell function, we analyzed proliferation and apoptosis of splenocytes and lymph node cells from *Gadd45a*$^{-/-}$, *p21*$^{-/-}$, *Gadd45a*$^{-/-}$*p21*$^{-/-}$, and wt mice (Figure 3). The sensitivity of primary T cell activation in *Gadd45a*$^{-/-}$ and double null splenocytes was different from wt cells, with approximately a 3- to 10-fold shift in the dose-response curve to the left (Figure 3A). For example, naive spleen cells from wt mice proliferated poorly at an anti-CD3 concentration of 3 ng/ml, whereas the proliferation of naive *Gadd45a*$^{-/-}$ and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ splenic cells was approximately one-third of their maximal response. In addition to enhanced sensitivity, at their maximal response *Gadd45a*$^{-/-}$ splenocytes incorporated almost twice as much [^{3}H]thymidine as wt cells. In contrast, both the dose-response curve and the maximal proliferation of *p21*$^{-/-}$ splenocytes was similar to that of wt cells. Cells lacking both *Gadd45a* and *p21* did not proliferate more vigorously than *Gadd45a*$^{-/-}$ splenocytes. The hyperresponsive phenotype was also apparent with lymph node cells (Figure 3B) and was evident in both the CD4$^+$ (Figure 3C) and CD8$^+$ (see Supplemental Figure SA at http://www.immunity.com/cgi/content/full/16/4/499/DCI) subsets. Recently, a previous report has shown that p21 is involved in cytokine-induced proliferation (Balomenos et al., 2000). To determine whether Gadd45a is a regulator of cytokine-induced proliferation, we cultured fresh *Gadd45a*$^{-/-}$, *p21*$^{-/-}$, *Gadd45a*$^{-/-}$*p21*$^{-/-}$, and wt lymph node cells with different concentrations of IL-2 (Figure 3D). *Gadd45a*$^{-/-}$ T cells stimulated with IL-2 proliferated in a dose-dependent fashion to the same extent as wt cells. As reported, *p21*$^{-/-}$ T cells proliferated to a greater extent than either *Gadd45a*$^{-/-}$ T and wt cells. Moreover, *Gadd45a*$^{-/-}$*p21*$^{-/-}$ lymphocytes activated with IL-2 had a proliferative response similar to *p21*$^{-/-}$ T cells, demonstrating that p21 but not Gadd45a plays a role in the control of IL-2-induced proliferation. To determine whether the lack of Gadd45a or p21 affected TCR-independent activation, we analyzed the proliferative response of lymph node cells after stimulation with phorbol ester (PMA) and a calcium ionophore A23187 (Figure 3E). *Gadd45a*$^{-/-}$ and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ lymph node cells did not have a proliferative advantage with respect to wt cells following stimulation with PMA and A23187. These results suggest that Gadd45a specifically regulates T cell proliferation induced by the TCR signaling. Consistent with the hyperproliferative response after TCR activation, the analysis of the cell cycle distribution of anti-CD3-stimulated CD4$^+$ cells revealed a large decrease in the proportion of cells in G1 in *Gadd45a*$^{-/-}$ (44 ± 3%) and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ cells (42 ± 5%) with respect to wt (63 ± 4%) (Figure 3F). There was also an increase in S phase of *Gadd45a*$^{-/-}$ (40 ± 2%) and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ (41 ± 3%) cells compared with wt (26 ± 2%). Notably, there was no difference among these groups with respect to the number of hyplodiploid (apoptotic) cells, a finding that was confirmed after 4 days of stimulation as well (data not shown). These results indicate that it is increased cell division and not decreased apoptosis that accounts for the augmented [^{3}H]thymidine incorporation in the *Gadd45a*$^{-/-}$ and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ cells.

In view of the hyperproliferative response of

Table 3. Summary of Laboratory Findings Associated to Lupus-Like Syndrome in Female *Gadd45a*$^{-/-}$, *Gadd45a*$^{-/-}$*p21*$^{-/-}$, and Wt Mice

	Wt	*Gadd45a*$^{-/-}$	*Gadd45a*$^{-/-}$*p21*$^{-/-}$
Median lifespan (mo.)	22.5	15.9	10.7
Anti-dsDNA[a] (%)	14	59	78
Anti-ssDNA[b] (%)	16	64	84
Anti-histone[c] (%)	16	50	70
Leukopenia[d] (%)	7	47	73
Lymphopenia[e] (%)	7	40	53
Proteinuria (mg/dl)	50	150	220
Glomerulonephritis	−	+	+

[a] Percentage of positive mice for antibodies against double-stranded DNA.
[b] Percentage of positive mice for antibodies against single-stranded DNA.
[c] Percentage of positive mice for antibodies against histone.
[d] Percentage of mice with <4000 leukocytes/μl in blood.
[e] Percentage of mice with <1200 lymphocytes/μl in blood.

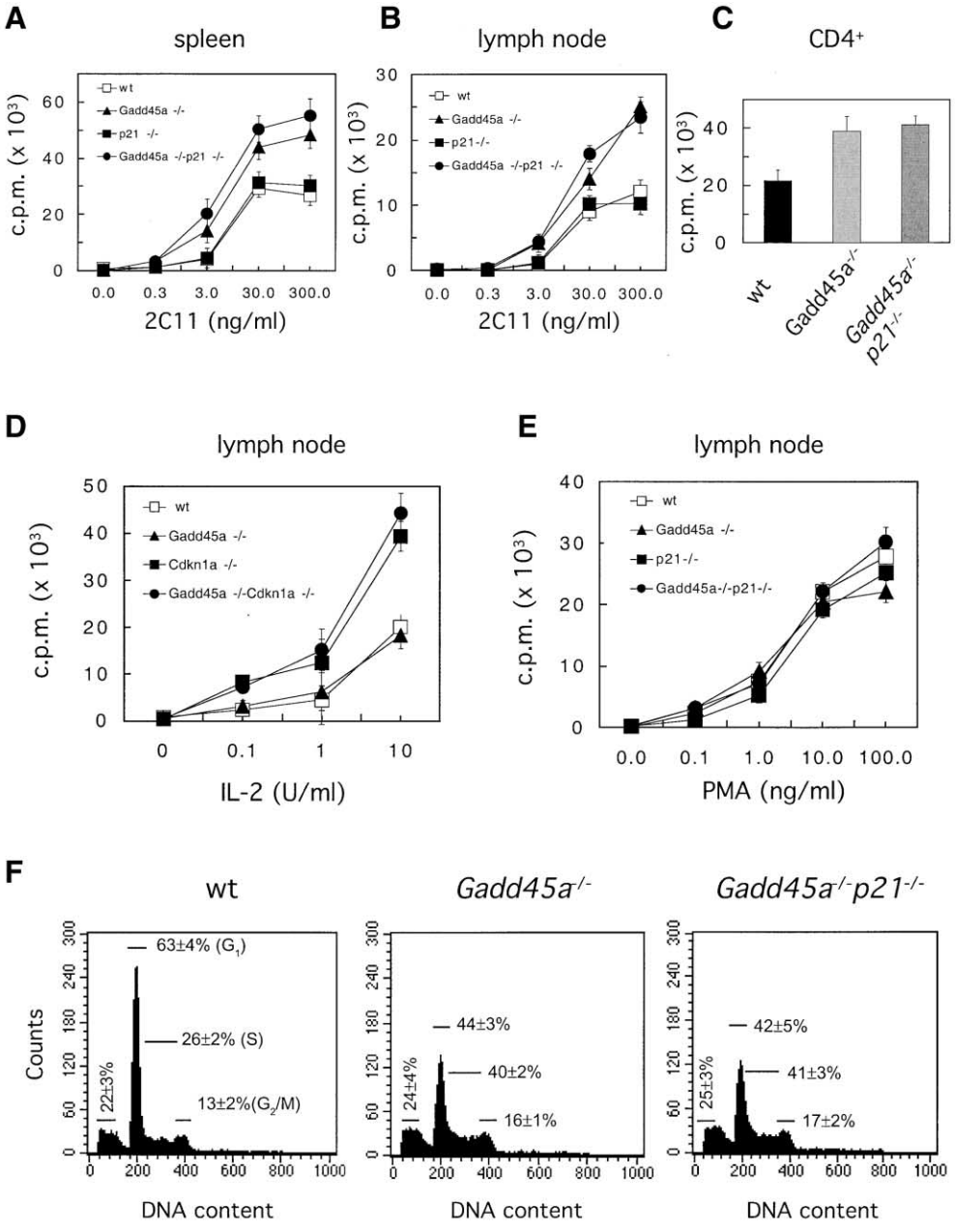

Figure 3. Proliferative Response of *Gadd45a*$^{-/-}$, *p21*$^{-/-}$, *Gadd45a*$^{-/-}$*p21*$^{-/-}$, and Wt Lymphocytes

(A) Increased proliferation of *Gadd45a*$^{-/-}$ and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ splenocytes. Splenic cells from 2-month-old wt (□), *Gadd45a*$^{-/-}$ (▲), *p21*$^{-/-}$ (■), and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ (●) mice were activated with an anti-CD3 antibody (2C11) and assayed for proliferation after 2 days. Proliferation was determined by [^{3}H] thymidine uptake in the last 16 hr of the assay. The results represent the mean ± SD of triplicate cultures of two experimental animals. Similar results were obtained in three independent experiments.

(B) Increased proliferation of *Gadd45a*$^{-/-}$ and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ lymph node cells. Wt (□), *Gadd45a*$^{-/-}$ (▲), *p21*$^{-/-}$ (■), and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ (●) lymph node cells were activated with an anti-CD3 antibody and assayed for proliferation as above.

(C) Increased proliferation in *Gadd45a*$^{-/-}$ CD4$^+$ cells and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ CD4$^+$ cells compared with wt CD4$^+$ cells following primary activation. CD4$^+$ cells were activated with 2C11 for 2 days and assayed for proliferation as described above.

(D) Hyperproliferative response of *p21*$^{-/-}$ and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ lymph node cells following IL-2 activation. Lymph node cells were activated with the indicated concentrations of IL-2 for 6 days. Cells were pulsed with [^{3}H] thymidine for the last 16 hr of the assay.

(E) Proliferative response of lymph node cells after PMA plus A23187 activation. Lymph node cells were activated with the same amount of A23187 (0.5 μM) and the indicated concentrations of PMA for 2 days and assayed for proliferation as above.

(F) Cell cycle analysis of CD4$^+$ cells from wt, *Gadd45a*$^{-/-}$, and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice. Following 2 days of activation with 2C11, CD4$^+$ cells were assayed for PI staining, and the FACS profiles are shown for one wt, one *Gadd45a*$^{-/-}$, and one *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mouse; percentages represent the mean ± SD, n = 4.

Table 4A. Splenocyte B Cells and T Cell Subsets in Wt, *Gadd45a*$^{-/-}$, and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ Mice

	Total ($\times 10^6$)[a]	B220+CD3−[b]	TCR[b]	CD4+[b]	CD8+[b]	CD44+[b]	CD62L+[b]	CD25+[b]	CD69+[b]
Wt	95.2 ± 4.5	67.6 ± 3.5	20.7 ± 1.2	52.0 ± 2.4	32.8 ± 4.3	76.4 ± 15.5	62.0 ± 20.4	14.2 ± 3.0	11.1 ± 2.7
Gadd45a$^{-/-}$	105.1 ± 5.5	65.6 ± 3.1	30.1 ± 2.7	56.2 ± 5.6	31.3 ± 4.3	73.8 ± 14.0	61.1 ± 16.9	9.1 ± 2.1	6.95 ± 2.26
Gadd45a$^{-/-}$ *p21*$^{-/-}$	103.5 ± 3.9	64.5 ± 4.7	27.5 ± 2.3	55.0 ± 7.9	32.9 ± 5.6	65.9 ± 12.5	50.0 ± 8.2	10.1 ± 4.0	8.8 ± 2.5

[a] Total Number of splenocytes (mean ± SD) from 2-month-old wt, *Gadd45a*$^{-/-}$, and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice.
[b] B cells and T cell subsets are given as percentages of total splenocytes (mean ± SD), n = 4.

Table 4B. Lymph Node B Cells and T Cell Subsets in Wt, *Gadd45a*$^{-/-}$, and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ Mice

	Total ($\times 10^6$)[a]	B220+CD3−[b]	TCR[b]	CD4+[b]	CD8+[b]	CD44+[b]	CD62L+[b]	CD25+[b]	CD69+[b]
Wt	3.6 ± 0.3	36.3 ± 2.2	55.1 ± 3.6	60.0 ± 7.2	30.2 ± 5.3	73.2 ± 13.6	93.0 ± 8.2	11.8 ± 2.0	11.2 ± 2.2
Gadd45a$^{-/-}$	4.3 ± 0.4	33.5 ± 1.6	64.7 ± 2.8	63.2 ± 6.5	32.2 ± 3.3	68.8 ± 13.9	90.3 ± 7.9	9.2 ± 2.2	10.4 ± 1.2
Gadd45a$^{-/-}$ *p21*$^{-/-}$	4.16 ± 0.4	31.5 ± 2.6	65.1 ± 3.1	65.2.0 ± 5.1	30.9 ± 4.6	67.3 ± 14.5	85.3 ± 10.2	13.3 ± 5.0	16.2 ± 1.5

[a] Total number of lymph node cells per lymph node (eight lymph nodes were collected per mouse) (mean ± SD) from 2-month-old wt, *Gadd45a*$^{-/-}$, and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice.
[b] B cells and T cell subsets are given as percentages of total lymph node cells (mean ± SD), n = 4.

Gadd45a$^{-/-}$ T cells in vitro, we analyzed the T cell composition in lymphoid tissues (Tables 4A and 4B and Supplemental Tables SB1, SB2, SC1, and SC2 at http://www.immunity.com/cgi/content/full/16/4/499/DCI). The proportion of total T cells, CD4$^+$, and CD8$^+$ were normal in the thymus of the *Gadd45a*$^{-/-}$ and double null mice (data not shown). However, in the secondary lymphoid tissues we noted a 50% increase in the proportion of total T cells in the spleen of *Gadd45a*$^{-/-}$ mice (30 ± 1.9%) with respect to wt (20.7 ± 1.2%) (Table 4A). This difference was maintained in the double null mice. The increase in total T cell number was due to an increase in both CD4$^+$ and CD8$^+$ cells. No differences in the CD4$^+$/CD8$^+$ ratio were noted among the different genotypes. Moreover, the proportion of total T cells in the lymph nodes of *Gadd45a*$^{-/-}$ mice (64.7 ± 2.8) and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ (65.1 ± 3.1) was also increased compared to wt (55.1 ± 3.6) (Table 4B). We have also examined the expression of surface activation markers such as CD44, CD62L, CD25, and CD69 in splenocytes and lymph node cells from 2-month-old *Gadd45a*$^{-/-}$ and *Gadd45a*$^{-/-}$*p21*$^{-/-}$; no differences were found in any of these parameters (Tables 4A and 4B and Supplemental Tables SB1, SB2, SC1, and SC2 at http://www.immunity.com/cgi/content/full/16/4/499/DCI). Therefore, the proliferative advantage of *Gadd45a*$^{-/-}$ T cells is not attributable to the presence of greater numbers of preactivated peripheral T cells.

To examine whether the lack of *Gadd45a* and/or *p21* affected B cell function, we analyzed the kinetics of B cell proliferation and B cell survival. No significant differences were noted in the [^{3}H]thymidine incorporation of purified B cells stimulated with anti-IgM or LPS (Figure 4A and Supplemental Figure SA at http://www.immunity.com/cgi/content/full/16/4/499/DCI). Additionally, we used PI staining as a measure of B

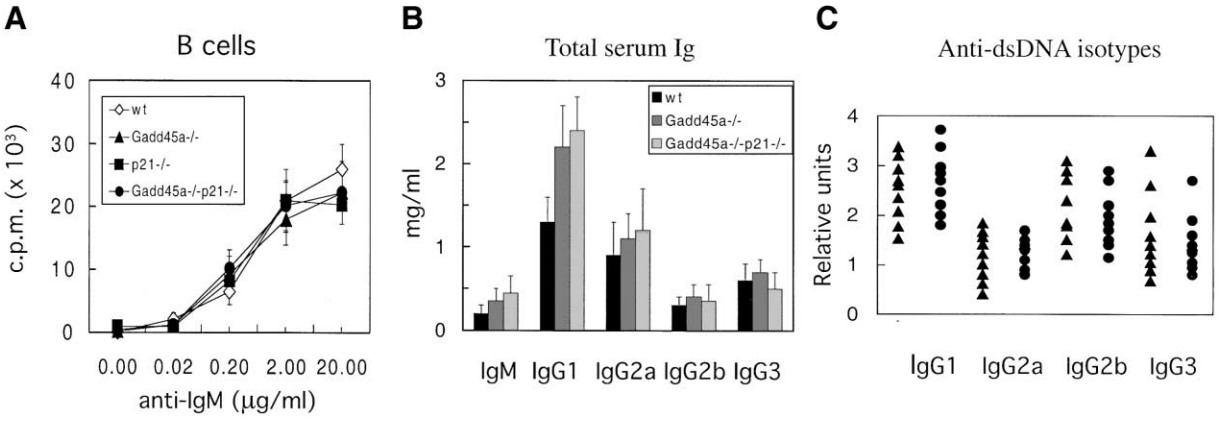

Figure 4. Proliferative Response of Purified B Cells, Total Serum Ig, and Anti-dsDNA Isotypes

(A) Proliferation of purified B cells from 2-month-old wt (□), *Gadd45a*$^{-/-}$ (▲), *p21*$^{-/-}$ (■), and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ (●) mice following anti-IgM activation. B cells were activated with anti-IgM and assayed for proliferation after 2 days. Proliferation was determined by [^{3}H] thymidine uptake in the last 16 hr of the assay. The results represent the mean ± SD of triplicate cultures of two experimental animals.
(B) Serum concentrations of IgM and IgG isotypes from *Gadd45a*$^{-/-}$, *Gadd45a*$^{-/-}$*p21*$^{-/-}$, and wt mice. Data represent the mean ± SD, n = 10.
(C) Level of anti-dsDNA IgG isotypes of *Gadd45a*$^{-/-}$ (▲) and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ (●) mice, n = 10.

cell apoptosis after IgM stimulation and observed no differences between the different genotypes (data not shown). Furthermore, we found that the percentage of B cells in the spleen and lymph nodes from 2-month-old *Gadd45a*$^{-/-}$ and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice was similar to that of wt animals (Tables 4A and 4B). These results suggest that neither *Gadd45a* nor *p21* is involved in the control of B cell stimulation or apoptosis.

Analysis of total serum Ig from 7- to 9-month-old *Gadd45a*$^{-/-}$ and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice showed hypergammaglobulinemia (Figure 4B). *Gadd45a*$^{-/-}$ and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice had a 2-fold increase in the levels of IgM and IgG1 with respect to wt mice. Seven- to nine-month-old *Gadd45a*$^{-/-}$ and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice developed greater concentrations of IgG1 anti-dsDNA antibodies (Figure 4C). Moreover, six of ten *Gadd45a*$^{-/-}$ mice and five of ten *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice developed high concentrations of IgG2b and IgG3 anti-dsDNA antibodies.

Discussion

The main findings of this study are: (1) *Gadd45a* is a negative regulator of T cell proliferation following TCR-mediated activation, (2) lack of *Gadd45a* leads to the spontaneous development of a lupus-like syndrome characterized by high titers of autoantibodies against dsDNA, ssDNA, and histone, severe leukopenia, lymphopenia, proteinuria, and autoimmune glomerulonephritis that cause premature death in female mice, (3) as reported (Balomenos et al., 2000), *p21* is a negative regulator of cytokine-induced T cell proliferation, and (4) lack of both p53-effector genes enhances the hyperproliferative response of T cells and accelerates dramatically the development of disease.

Gadd45a$^{-/-}$ T cells have a lower threshold for activation and a proliferative advantage compared to wt cells following anti-CD3 activation. In contrast, *p21*$^{-/-}$ T cells had a hyperproliferative response only after sustained IL-2 stimulation. Consistent with this, lack of both genes had no further effect on the hyperproliferation of cells deficient in Gadd45a or p21 when they were activated with anti-CD3 or IL-2, respectively. These findings strongly suggest that the p53-effector genes *Gadd45a* and *p21* have different roles in controlling immune responses.

It has been reported that *Gadd45g*$^{-/-}$ T_H1 effector cells are less sensitive to activation-induced cell death than wt cells (Lu et al., 2001). The increased proliferation in *Gadd45a*$^{-/-}$ T cells could be due to an increase in T cell survival after T cell activation. However, neither *Gadd45a* nor *p21* is involved in T cell apoptosis induced by anti-CD3 or B cell apoptosis induced by IgM. In addition, we have previously reported that *Gadd45a*-deficient thymocytes die normally after exposure to ionizing radiation, which requires p53, as well as after exposure to glucocorticoids (Hollander et al., 1999). Therefore, we conclude that Gadd45a function is specific for the control of T cell proliferation but not apoptosis. Interestingly, two different reports indicate that *Gadd45g* does not play a critical role in the control of T cell proliferation (Hoffmeyer et al., 2001; Lu et al., 2001) and/or genomic instability (Hoffmeyer et al., 2001). CD4$^+$ T cells from *Gadd45g* mice did not show a hyperproliferative response following anti-CD3 activation (Lu et al., 2001). Our findings indicate that *Gadd45a* has a different role than that of *Gadd45g* in the immune system. Although both genes contribute to control T cell growth, *Gadd45a* is a negative regulator of T cell proliferation whereas *Gadd45g* may induce apoptosis of T_H1 cells in vitro. Generation of *Gadd45b*$^{-/-}$ mice will probably be required to determine the role of this Gadd45 family member in immune regulation, but it has already been implicated in cytokine signaling in T cells (Yang et al., 2001).

While Gadd45a has been implicated in growth control, our results indicate that *Gadd45a*$^{-/-}$ T cells do not have a generalized cell cycle control defect. In particular, the proliferative response of *Gadd45a*$^{-/-}$ and double null cells after PMA plus A23187 was equivalent to wt cells (Figure 3E). This indicates that the hyperproliferative response in *Gadd45a*$^{-/-}$ cells requires TCR activation because we only find this difference after anti-CD3 stimulation. If the cause of the hyperproliferative response in *Gadd45a*$^{-/-}$ cells was due to a fundamental problem in cell cycle control, then we should expect a hyperproliferative phenotype regardless of whether T cell activation was achieved with anti-CD3 or PMA plus A23187. In addition, B cell proliferation was also equivalent to wt cells (Figure 4A). It should also be pointed out that, unlike p21, Gadd45 has not been found to interact with or affect factors controlling G1 cell cycle progression. For example, no interaction with G1 cdk or cyclin proteins has been found (Smith et al., 1994; Zhan et al., 1999), G1 checkpoint activation was normal in *Gadd45a* null embryo fibroblasts (Hollander et al., 1999), and Gadd45a did not inhibit G1 Cdk activities in vitro (Smith et al., 1994; Zhan et al., 1999).

There is much evidence supporting the notion that T cell activation has an important role in murine and human autoimmunity. Neonatal thymectomy markedly ameliorates the lupus-like disorder in MRL and NZB/W F1 mice (Hang et al., 1984). Depletion of T cells and treatment with antibodies to Thy-1 or CD4 suppress the production of anti-dsDNA antibodies and ameliorate the disease in NZB/W F1 mice and human lupus (Datta et al., 1987; Wofsy and Seaman, 1985). Although the mechanism by which CD4$^+$ cells influence B cells in the production of anti-dsDNA antibodies in lupus is unknown, it is now clear that polyclonal CD4$^+$ T cell activation is characteristic of spontaneous murine and human disease (Rozzo et al., 1994). Although the causes of loss of tolerance to self are unknown, there is growing evidence that abnormalities in T cell proliferation can contribute to this process. An elegant report has demonstrated that naive CD4$^+$ cells from autoimmune-prone mice are hyperproliferative compared to controls and that such T cells have a lower threshold of activation to antigens with a low affinity for TCR (Vratsanos et al., 2001). In addition, Balomenos et al. (2000) found that T cells from *p21*$^{-/-}$ mice exhibit significant proliferative advantage compared with wt cells, suggesting that following repeated encounters with autoantigen, autoreactive *p21*-deficient T cells hyperproliferate and provoke the break in tolerance. We propose that the hyperproliferative response in *Gadd45a*$^{-/-}$ CD4$^+$ cells, together with the lower threshold for activation, are sufficient to provoke breaking of peripheral tolerance in these animals. We

are currently exploring the possibility that a defect in central tolerance (i.e., in thymocyte selection) might also contribute to this phenotype.

We have noted many similarities between the clinical signs of *Gadd45a*$^{-/-}$ mice and human SLE. First, this lupus-like syndrome affects females more severely than males. Only *Gadd45a*$^{-/-}$ females develop high titers of autoantibodies against dsDNA, ssDNA, and histone, leukopenia, lymphopenia, and autoimmune glomerulonephritis. Moreover, lack of *p21* increases the susceptibility to develop a lupus-like syndrome (Balomenos et al., 2000). Interestingly, by the generation of the *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice we show that the lack of both genes dramatically accelerates the development of the disease. The earlier development of autoantibodies in the double null mice, with respect to *Gadd45a*$^{-/-}$ or *p21*$^{-/-}$ mice together with exacerbated leukopenia, lymphopenia, proteinuria, and decreased lifespan, demonstrates that the lack of both genes has an additive effect in the development of the autoimmune disease in vivo. Therefore, the in vitro and in vivo findings indicate that both genes have different functions in the negative regulation of T cell proliferation and that lack of either can lead to the development of a lupus-like syndrome. Since a variety of viral and cellular factors are known to interfere with normal p53 function (Prives and Hall, 1999), aberrant expression of one or more of such factors in T cells in vivo would be expected to perturb the normal expression of *Gadd45a* and *p21* and could well contribute to the development of lupus clinically.

Experimental Procedures

Mice

NIH is an AALAC accredited animal facility, and all experiments were done under an approved NCI animal study protocol. Mice were housed in plexiglass cages and given autoclaved NIH 31 diet and water ad libidum. Mice were observed three times per week for ill health, and those who were moribund, cachectic, or nonresponsive were sacrificed for necropsy. Tissues were taken for histopathological analysis, sectioned, stained with hematoxylin and eosin, and evaluated by a board-certified veterinary pathologist. *Gadd45a*$^{-/-}$ and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice were generated in our laboratory as described previously (Hollander et al., 1999). *p21*$^{-/-}$ mice were obtained from the laboratory of C. Deng (Deng et al., 1995). MRL-lpr mice were obtained from Jackson Laboratories (Bar Harbor, ME).

T and B Cell Proliferation and Apoptosis

Spleen cells and lymph node cells were isolated by gentle disruption of the tissues (6- to 8-week-old mice) with the flat end of a syringe against the bottom of a 70 μm nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ). The proliferation assay was carried out in 96-well flat-bottom microtiter plates. Spleen cells or lymph node cells (1×10^5/well) were cultured in RPMI-1640 (Gibco BRL, Grand Island, NY) supplemented with 4 mM glutamine, 5×10^{-5} M β-mercaptoethanol, 100 U/ml penicillin, 150 μg/ml gentamicin, and 10% heat-inactivated FCS. When indicated, soluble anti-CD3 (145-2C11, PharMingen, San Diego, CA) was added at different concentrations. Proliferation was quantified after 48 hr of activation with 2C11. The wells were pulsed with 1 μCi [^{3}H]thymidine, and 16 hr later the wells were harvested and the incorporation of radioactivity was determined on scintillation counter (Beckman, Palo Alto, CA). CD4$^+$, CD8$^+$, and B cells were isolated from spleens of 6-to 8-week-old mice by negative selection using affinity chromatography immunocolumns (Cytovax, Alberta, Canada) following the manufacturer's instructions. Isolated CD4$^+$ or CD8$^+$ cells (1×10^6/ml) were stimulated with anti-CD3 in the presence of irradiated spleen cells (0.5×10^6/ml). After 48 hr, cells were collected for determination of [^{3}H]thymidine incorporation and stained with PI for cell cycle analysis and apoptosis. For B cell proliferation assay, purified B cells (1×10^6/ml) were activated with goat F(ab′)2 anti-mouse IgM (2 μg/ml, Jackson ImmunoResearch, West Grove, PA) or LPS (20 μg/ml, Sigma, St. Louis, MO) for 48 hr. Proliferation was measured by uptake of [^{3}H]thymidine in the last 16 hr of pulse.

ELISA

Presence of autoantibodies directed against dsDNA, ssDNA, and histone were examined in the mice serum by ELISA as described in Isenberg et al. (1987). In brief, 96-well flat-bottom plates (Immulon 4HBX, Dynex Technologies) were precoated with 2.5 μg of poly-L-lysine (Sigma) for 1 hr at 37°C. Plates were incubated with 0.5 μg/well of dsDNA, ssDNA (denatured calf thymus DNA by boiling 10 and 5 min on ice), or histone type II-S from calf thymus (Sigma) overnight at 4°C. After blocking the nonspecific sites with 5% FCS for 1 hr at 37°C, mice serum samples (serial dilution from 1/50 to 1/31250) were added for 3 hr at room temperature. Then followed 1 hr of incubation with HRP-labeled goat anti-mouse IgG Fc-specific (Jackson Immunoresearch) diluted 1:500. A substrate solution containing equal volumes of ABTS peroxidase substrate system solutions A and B (Kirkegaard and Perry Laboratories, Gaithesburg, MD) was added, and the plates were read at 405 nm in ELISA reader (Emax Molecular Device). Pooled positive serum derived from four 4-month-old MRL-lpr mice and pooled serum from four wt mice were used as positive and negative controls, respectively. Results were expressed relative to the ELISA absorbance produced by a standard of pooled MRL-lpr serum which was assigned an arbitrary value of 100 units and was considered positive at ten units or more. Antibodies against dsDNA were also detected by immunofluorescence using the Crithidia luciliae test. Slides with Crithidia were purchased from DiaSorin Inc. (Stillwater, MN) and incubated with the serum according to the manufacturer's protocol. Serum antibody data were compared by Wilcoxon test using JMP 3.2 software.

Flow Cytometry Analysis

Isolated lymph node cells and spleen cells ($0.5–1 \times 10^6$) were washed in staining buffer (PBS with 5% bovine serum albumin and 0.1% NaN_3) and pelleted. To block the nonspecific binding sites, the cells were incubated with 10 μl of an antibody specific for mouse Fc receptor (2.4G2, PharMingen, San Diego, CA). After 10 min incubation, 10 μl of monoclonal antibody at the appropriate dilution was added and incubated for 30 min at 4°C. Finally, the cells were resuspended in staining buffer and analyzed with FACSCalibur (Beckton Dickinson). All the antibodies used in this study, CD4, CD8, B220, CD69, CD45RB, CD25, CD69L, and CD44, were purchased from PharMingen, San Diego, CA. PI staining was used to quantify proportion of apoptotic cells. Cell cycle distribution was analyzed by using the ModFit software.

Histopathology and Immunohistochemistry

Kidneys from *Gadd45a*$^{-/-}$ and *Gadd45a*$^{-/-}$*p21*$^{-/-}$ mice were fixed in formalin (Anatech, Battle Creek, MI) and embedded in paraffin. Three micron thick sections, stained with periodic acid-Schiff reagent (PAS), were examined by light microscopy. Mouse kidneys were embedded in Tissue-Tek (Sakura, Torrance, CA) and snapfrozen in isopentane. Five micron cryosections were fixed in cold acetone, briefly rehydrated, blocked with 5% normal goat serum, and incubated for 1 hr at room temperature with 1:200 dilution of Alexa-488-conjugated goat anti-mouse IgG (g chain), anti-mouse IgM (m chain), or anti-mouse IgA (a chain), all obtained from Molecular Probes (Eugene, OR) or fluoresceinated goat anti-mouse C3, obtained from ICN (Costa Mesa, CA). Sections were washed with PBS, mounted with glycerol-containing medium, and observed with a fluorescent microscope equipped with appropriate optics. A total of 26 animals were examined and divided into two age groups: young mice (28–35 weeks old) and old (35–84 weeks old). The young group included three wild-type animals (two female and one male), four *Gadd45a*$^{-/-}$ mice (two female and two male), and three *Gadd45a*$^{-/-}$*p21*$^{-/-}$ (all female). The old group included six wild-type (all females), seven *Gadd45a*$^{-/-}$ (all females), and three *Gadd45a*$^{-/-}$*p21*$^{-/-}$ (all females). Kidney sections were examined by two investigators masked to the identity of the tissues.

Statistical Analysis

The differences between experimental groups of serum antibody were compared for significance by the Wilcoxon test using JMP 3.2 software.

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