Gadd45β and Gadd45γ are critical for regulating autoimmunity

Lin Liu,[1] Elise Tran,[2] Yani Zhao,[1] Yuchen Huang,[1] Richard Flavell,[2,3] and Binfeng Lu[1]

[1]Department of Immunology, University of Pittsburgh School of Medicine, E1047 BST, Pittsburgh, PA 15261
[2]Section of Immunobiology and [3]Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT 06520

The number of effector T cells is controlled by proliferation and programmed cell death. Loss of these controls on self-destructive effector T cells may precipitate autoimmunity. Here, we show that two members of the growth arrest and DNA damage-inducible (*Gadd45*) family, β and γ, are critical in the development of pathogenic effector T cells. CD4$^+$ T cells lacking *Gadd45β* can rapidly expand and invade the central nervous system in response to myelin immunization, provoking an exacerbated and prolonged autoimmune encephalomyelitis in mice. Importantly, mice with compound deficiency in *Gadd45β* and *Gadd45γ* spontaneously developed signs of autoimmune lymphoproliferative syndrome and systemic lupus erythematosus. Our findings therefore identify the Gadd45β/Gadd45γ-mediated control of effector autoimmune lymphocytes as an attractive novel target for autoimmune disease therapy.

CORRESPONDENCE
Richard Flavell:
richard.flavell@yale.edu
OR
Binfeng Lu:
Binfeng@pitt.edu

Abbreviations used: AICD, activation-induced cell death; CFSE, carboxyfluorescein succinimidyl ester; CNS, central nervous system; dsDNA, double-stranded DNA; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; PAMP, pathogen-associated molecular pattern.

Gadd45β and Gadd45γ are critical for the initiation of the Th1 type response and they function as part of the immune response that combats intracellular pathogens. *Gadd45β* expression is induced in DCs and macrophages by pathogen-associated molecular patterns (PAMPs) such as LPS (1). In addition, *Gadd45β* and *Gadd45γ* are induced in CD4$^+$ T cells by TCR signaling or inflammatory cytokines, such as IL-12 and IL-18 (2–4). Moreover, Gadd45β and Gadd45γ are required for functions of DCs, NK cells, and T cells (2–4). The functional role of Gadd45β and Gadd45γ in CD4$^+$ T cells is attributed to their abilities to directly amplify and prolong the activation of p38 MAP kinase (2–4).

Besides their role in mediating the stress responses, genes of the *Gadd45* family play important roles in cell cycle and programmed cell death. Proteins of the Gadd45 family inhibit proliferation of cells by directly interacting with members of the cell cycle machinery such as Cdc2 and cyclin B (5, 6). Proteins of the Gadd45 family promote apoptosis in some cellular contexts by activating JNK/p38 MAP kinases (4, 7–9).

In this paper, we provide evidence that Gadd45β regulates the homeostasis of CD4$^+$ T cells. T cells lacking *Gadd45β* proliferated faster than wild-type controls and were much more resistant to activation-induced cell death (AICD) in vitro. Deletion of *Gadd45β* also resulted in exacerbation of organ-specific autoimmune disease experimental autoimmune encephalomyelitis (EAE). Furthermore, *Gadd45β*×*Gadd45γ* compound deficiency led to a severe lymphoproliferative disorder with signs of systemic autoimmune diseases such as autoimmune lymphoproliferative syndrome and systemic lupus erythematosus.

L. Liu, E. Tran, and B. Lu contributed equally to this work.

RESULTS

Gadd45β regulates T cell proliferation and AICD

To determine whether Gadd45β is involved in cell proliferation in T cells, we stimulated CD4$^+$ T cells isolated from wild-type and *Gadd45β*-deficient animals with APCs and anti-CD3 for 3 d. At the beginning of each experiment, cells were stained with carboxyfluorescein succinimidyl ester (CFSE) to facilitate the monitoring of cell division. *Gadd45β*-deficient CD4$^+$ T cells underwent slightly more cell divisions than wild-type CD4$^+$ T cells (Fig. 1 A). In addition to naive CD4$^+$ T cells, we also examined whether Gadd45β regulated proliferation of effector T cells stimulated with anti-CD3. Compared with wild-type T cells, *Gadd45β*-deficient Th1 cells underwent more cell divisions when stimulated with plate-bound anti-CD3 (Fig. 1 B).

IL-12 and IL-18 have also been shown to induce Gadd45β expression (2). To test whether

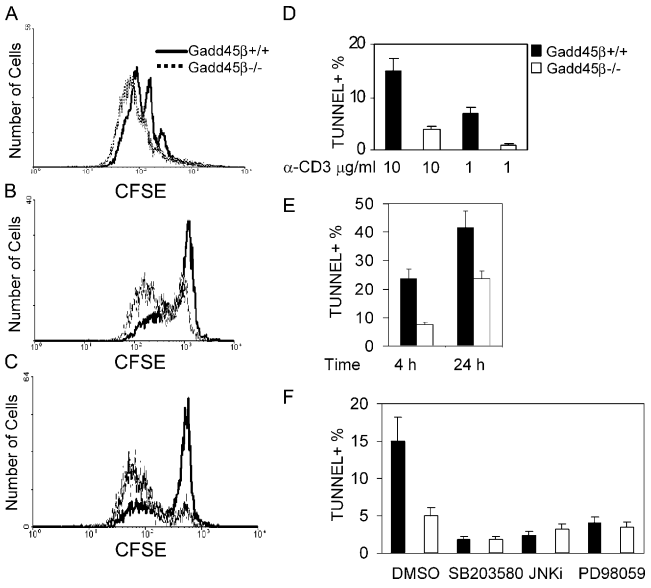

Figure 1. *Gadd45β* deficiency led to hyperproliferation and resistance to AICD. (A) Naive $CD4^+$ T cells from wild-type and $GADD45\beta^{-/-}$ mice were isolated, stained with CFSE, and cultured in nonpolarizing conditions with wild-type T cell–depleted irradiated splenocytes from wild-type animals as antigen-presenting cells. 3 d later, cell division was analyzed by flow cytometry. (B) Effector Th1 cells were stained with CFSE and stimulated on 1 μg/ml of plate-bound anti-CD3 and 1 μg/ml anti-CD28 for 48 h. Cell division was analyzed by flow cytometry. (C) Effector Th1 cells were stained with CFSE and stimulated with 3.4 ng/ml IL-12 and 10 ng/ml IL-18 for 48 h. Cell division was analyzed by flow cytometry. (D) Effector Th1 cells were stimulated on plate-bound anti-CD3 (dose as labeled) for 6 h. Apoptosis was measured by TUNNEL assay and analyzed by flow cytometry. (E) Effector Th1 cells were stimulated on plate-bound anti-Fas (clone Jo2) for 4 and 24 h as indicated. Apoptosis was measured by TUNNEL assay and analyzed by flow cytometry. (F) Effector Th1 cells were stimulated on plate-bound anti-CD3 (10 μg/ml) in the presence of various chemical inhibitors as indicated for 6 h. Apoptosis was measured by TUNNEL assay and analyzed by flow cytometry. Data are representative of five independent experiments. One wild-type mouse and one *Gadd45β*-deficient mouse were used in each experiment.

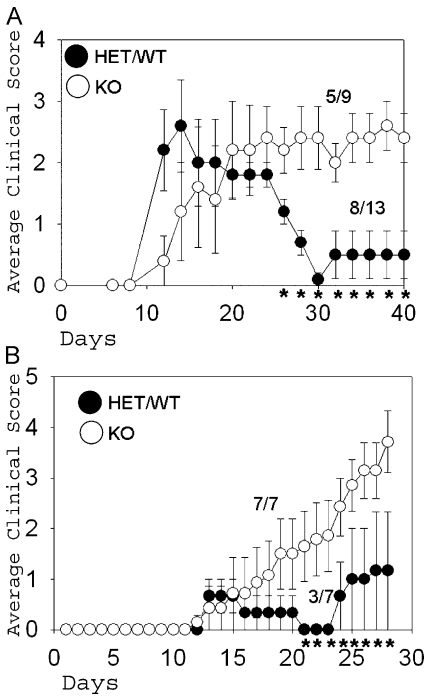

Figure 2. *Gadd45β* deficiency led to exacerbation of EAE. (A) Wild-type mice and *Gadd45β*$^{-/-}$ littermates that had been backcrossed onto C57BL/6 background for six generations were immunized with MOG_{35-55} peptide to elicit EAE. Clinical signs and scores were recorded as described in Materials and methods. Data are the average clinical scores from mice that have shown clinical signs of EAE. (B) 3×10^6 $CD4^+$ T cells isolated from wild-type and littermate *Gadd45β*-deficient mice were injected in the tail vein of *Rag1*$^{-/-}$ recipient mice. After 24 h, *Rag1*$^{-/-}$ recipients were immunized with MOG_{35-55} peptide to elicit EAE. Data are the average clinical scores from mice that have shown clinical signs of EAE. *, $P < 0.05$, determined by Mann-Whitney statistics.

Gadd45β regulates cytokine-driven proliferation, we stimulated Th1 cells from wild-type mice and *Gadd45β*-deficient mice with IL-12 and IL-18. We observed that *Gadd45β*-deficient Th1 cells divided more than wild-type Th1 cells in response to IL-12 and IL-18 (Fig. 1 C). Therefore, Gadd45β controls T cell proliferation in response to either TCR or inflammatory signals.

Gadd45γ was shown to mediate AICD in Th1 cells (4). Therefore, we examined whether Gadd45β is also required for AICD. Both wild-type and *Gadd45β*-deficient Th1 cells were stimulated with plate-bound anti-CD3 for 6 h, and apoptosis was measured by the TUNNEL assay. *Gadd45β*-deficient Th1 cells were much more resistant to AICD than wild-type Th1 cells (Fig. 1 D). Because Gadd45β potentiates p38 activation and p38 regulated the expression of FasL (10), the resistance to apoptosis by *Gadd45β*-deficient $CD4^+$ T cells could be due to diminished surface expression of FasL. Surprisingly, we have not observed any difference in the surface expression of Fas and FasL between wild-type Th1 cells and *Gadd45β*-deficient Th1 cells (unpublished data). In addition, *Gadd45β*-deficient Th1 cells were more resistant to AICD triggered by an anti-Fas antibody than wild-type Th1 cells (Fig. 1 E). These data suggest that Gadd45β mediates death signaling inside cells. Because MAP kinases have been shown to mediate cell death by directly modifying proteins of the death machineries (for review see reference 11), Gadd45β can promote cell death by potentiating the activation of MAP kinases such as p38, JNK, and ERK. Consistent with this notion, chemical inhibitors of MAP kinases inhibited AICD in wild-type T cells to a low level comparable to the level observed in *Gadd45β*-deficient T cells (Fig. 1 F).

Gadd45β deficiency results in exacerbated and prolonged EAE in mice

Myelin-specific Th1 cells and their infiltration into the parenchyma of white matters are critical for the clinical course of EAE. Because Gadd45β is important for both the generation of Th1 cells (3, 12) and the elimination of hyper-activated

Table I. MOG-induced EAE in Gadd45β$^{+/+}$ or $^{-/-}$ mice with 50 μg of heat-inactivated *M. tuberculosis*

Genotype	*n*	Incidence of EAE	Mean maximal clinical score	Mean day of onset	Moribund/ death
		%			%
Gadd45β$^{+/+}$	13	62	3.1 ± 0.9	14.4 ± 3.3	0
Gadd45β$^{-/-}$	9	56	3.0 ± 1.0	17.6 ± 4.3	0

Table III. MOG-induced EAE in RAG1$^{-/-}$ recipients of Gadd45β$^{+/+}$ or $^{-/-}$ T cells

Donor T cells	*n*	Incidence of EAE	Mean maximal clinical score	Mean day of onset	Moribund/ death
		%			%
Gadd45β$^{+/+}$	7	43	1.8 ± 1.4	16.7 ± 6.4	14
Gadd45β$^{-/-}$	7	100	3.7 ± 1.6[a]	18.7 ± 4.2	71

[a]$P < 0.05$; determined by Mann-Whitney statistics.

Th1 cells (Fig. 1; reference 12), we decided to determine how Gadd45β is involved in the clinical course of Th1-mediated autoimmune disease EAE. To induce EAE, mice were immunized with myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ peptide in CFA. Wild-type mice developed a severe paralytic disease and recovered almost fully afterwards (Fig. 2 A; Table I). In contrast, *Gadd45β*$^{-/-}$ C57Bl/6 mice manifested a similar onset of the disease but sustained a longer duration of peak disease than wild-type mice (Fig. 2 A; Table I). No mice died due to suffering from severe EAE in this experiment (Table I). In contrast, when mice were immunized with $MOG_{35–55}$ in a higher dose of heat-inactivated *Mycobacterium tuberculosis* in 500 μg/mouse CFA, 60% *Gadd45β*$^{-/-}$ mice died of severe EAE, whereas none of the wild-type mice did (Table II). These data suggest that Gadd45β is required for suppression of EAE, likely due to its antiproliferative and pro-apoptotic functions. To confirm that such EAE severity was due to abnormal *Gadd45β*$^{-/-}$ T cell behavior, we transferred 3×10^6 naive *Gadd45β*$^{+/+}$ or $^{-/-}$ CD4$^+$ T cells into Rag1-deficient recipient mice, which were subsequently immunized with $MOG_{35–55}$/CFA. All recipients of *Gadd45β*$^{-/-}$ T cells developed clinical signs of EAE, whereas only 43% recipients of wild-type T cells did so after immunization (Table III). Furthermore, recipients of *Gadd45β*$^{-/-}$ T cells became moribund and were killed for analyses or ethical reasons, whereas recipients of wild-type T cells exhibited a much milder clinical course of disease (Table III; Fig. 2 B). Thus, *Gadd45β* deletion in T cells resulted in exacerbation of EAE, suggesting that regulation of T cells by Gadd45β is critical for the recovery from autoimmune response.

Accumulation of *Gadd45β*-deficient Th1 cells in the central nervous system (CNS)

The severe clinical signs of *Gadd45β*-deficient CD4$^+$ T cell recipient *Rag1*$^{-/-}$ mice were accompanied by a marked leukocyte infiltration and demyelination of the CNS tissues (Fig. 3 A). RT-PCR analysis revealed high levels of IFNγ mRNA (Fig. 3 B), but no detectable IL4 mRNA (not depicted) in the inflamed CNS of *Gadd45β*-deficient CD4 T cell recipient *Rag1*-deficient mice, supporting a Th1-type inflammation. In *Rag1*-deficient recipient mice, Th1 cells have been shown to induce EAE with a preferential recruitment of mononuclear cells, whereas Th2 cells promote a dominant polymorphonuclear cell infiltration (13). In immune-competent mice, it was found that IFN-γ is pivotal for shaping the leukocyte recruitment in the inflamed CNS; its function stimulates a mononuclear cell infiltration, whereas in its absence polymorphonuclear cells are predominantly recruited (14, 15). Based on morphological analysis of the CNS tissues from *Gadd45β*-deficient CD4$^+$ T cell recipient mice, the infiltrating leukocytes were predominantly mononuclear cells. In addition, flow cytometric examination of the infiltrating cells showed a typical FSC/SSC profile of mononuclear cells (Fig. 3 C). Indeed, high numbers of activated CD4$^+$ T cells, macrophages, and microglial cells (CD11b$^+$) were recovered from the CNS of *Gadd45β*-deficient T recipient mice with EAE (Fig. 3, C–E). To determine that the infiltrated CNS did contain MOG-specific CD4$^+$ T cells, we restimulated CNS-infiltrated cells ex vivo with $MOG_{35–55}$ peptides and measured their cytokine production by flow cytometry analysis of intracellular cytokine staining. More *Gadd45β*-deficient T cells than wild-type T cells produced IFN-γ in the recall response to $MOG_{35–55}$ (Fig. 3 D). Interestingly, we found that unlike the CNS, the spleens of *Gadd45β*$^{+/+}$ or $^{-/-}$ T cell recipient mice contained similar numbers of MOG-specific Th1 cells (Fig. 3 F). Thus, exacerbation of EAE was associated with a rapid accumulation of effector *Gadd45β*-deficient Th1 cells in the target CNS. These findings were surprising in light of our previous observations that Gadd45β deletion led to attenuated Th1 responses in vitro and in mice during *Listeria monocytogene* infection (3). We think that Gadd45β, being able to directly inhibit effector T cell proliferation and promote their apoptosis, is pivotal here in terminating a Th1 autoimmune response in a target tissue. In addition to being critical for the generation of Th1 responses against infection, Gadd45β regulates the duration of a Th1 cell–mediated autoimmune response.

Table II. MOG-induced EAE in Gadd45β$^{+/+}$ or $^{-/-}$ mice with 500 μg of heat-inactivated *M. tuberculosis*

Genotype	*n*	Incidence of EAE	Mean maximal clinical score	Mean day of onset	Moribund/ death
		%			%
Gadd45β$^{+/+}$	9	89	3.8 ± 0.5	13.6 ± 3.3	0
Gadd45β$^{-/-}$	10	90	4.3 ± 0.7	13.1 ± 2.1	60

Gadd45β/Gadd45γ double deficiency led to a lymphoproliferative disorder in older mice

In addition to their roles in preventing organ-specific autoimmunity, programmed cell death and proliferation play a critical role in regulating homeostasis of the immune system. Because

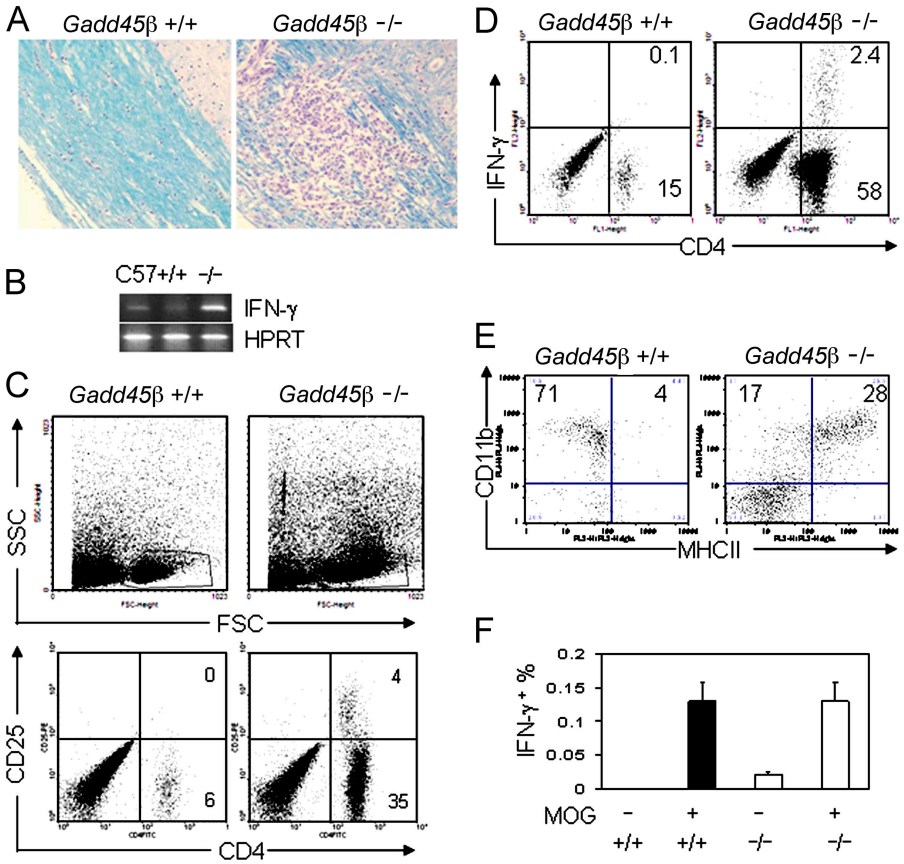

Figure 3. *Gadd45β* deficiency leads to severe inflammation in the later stage of EAE. 3×10^6 $CD4^+$ T cells isolated from wild-type and littermate *Gadd45β*-deficient mice were injected in the tail vein of $Rag1^{-/-}$ recipient mice. After 24 h, $Rag1^{-/-}$ recipients were immunized with MOG_{35-55} peptide to elicit EAE. (A) On day 28, to reveal T cell infiltration, spinal cords were isolated and analyzed by hematoxylin and eosin staining. (B) Total RNAs were purified and subjected to RT-PCR analysis for the expression of *IFN-γ* using the primer pair (5′-CATTGAAAGCCTAGAAAGTCTG-3′ and 5′-CTCATGAATGCATCCTTTTTCG-3′, controlled by *HPRT* (5′-GTTGGATACAG-GCCAGACTTTGTTG-3′ AND 5′-GAGGGTAGGCTGGCCTATAGGCT-3′). Total RNA from the CNS isolated from a naive C57BL/6 (C57) mouse was used as a control. (C) CNS mononuclear cells were isolated and analyzed by flow cytometry for $CD4^+$ T cell activation marker CD25. (D) Mononuclear cells isolated from the CNS were incubated with MOG_{35-55} peptide at 10 μg/ml for 4 h and the IFN-γ protein was analyzed by intracellular cytokine staining followed by flow cytometry. (E) Activation of $CD45^+Mac1^+$ macrophages/microglia was assayed by surface staining of MHC II. (F) The frequency of MOG-specific $CD4^+$ T cells in the spleen of the same mouse was determined by ex vivo stimulating splenocytes with MOG_{35-55} peptide and assayed using intracellular cytokine staining and followed by flow cytometry.

Gadd45β and Gadd45γ both regulated death and proliferation of $CD4^+$ T cells (12), we decided to examine whether deletion of these genes affected the homeostasis of the immune system. Cells from spleens and lymph nodes of young *Gadd45β*-deficient, *Gadd45γ*-deficient, and *Gadd45β/Gadd45γ* double-deficient mice were indistinguishable from those of young wild-type mice. In contrast, modest splenomegaly developed in *Gadd45β*-deficient mice when aged 10 mo old (Fig. 4 A). Strikingly, 10-mo-old *Gadd45β/Gadd45γ* double-deficient mice developed much more severe splenomegaly (Fig. 4 A). The enlargement of spleens was caused by an increase in the number of splenocytes (Fig. 4 B). This increase was largely due to accumulation of $CD4^+$ T cells and B cells (Fig. 4 C). $CD4^+$ T cells in the $Gadd45\beta^{-/-} \times Gadd45\gamma^{-/-}$ mice were predominantly effector/memory cells (Fig. 4 D). These data are consistent with the notion that Gadd45β and Gadd45γ synergistically regulate the homeostasis of preactivated $CD4^+$ T cells.

The fact that deletion of *Gadd45β* aggravated the induced organ-specific autoimmune disease EAE prompted us to examine whether Gadd45β and Gadd45γ controlled peripheral tolerance to autoantigens such as histones and double-stranded DNA (dsDNA). Sera were isolated from 10-mo-old *Gadd45β*-deficient and *Gadd45β×Gadd45γ* double-deficient mice, as well as wild-type mice and assayed for the presence of antihistone, anti-dsDNA, and total antibodies by ELISA. A large amount of antihistone and anti-dsDNA IgM and IgG1, but not IgG2a, was detected in sera from *Gadd45β×Gadd45γ* double-deficient mice, but not in *Gadd45β*-deficient or wild-type mice (Table IV). In addition, we have detected immune complex deposits in renal glomeruli of *Gadd45β×Gadd45γ* double-deficient mice, but not wild-type littermates (Fig. 4 e). Although we did not find high levels of antihistone and anti-dsDNA IgM and IgG1 in the sera of *Gadd45β*-deficient mice (unpublished

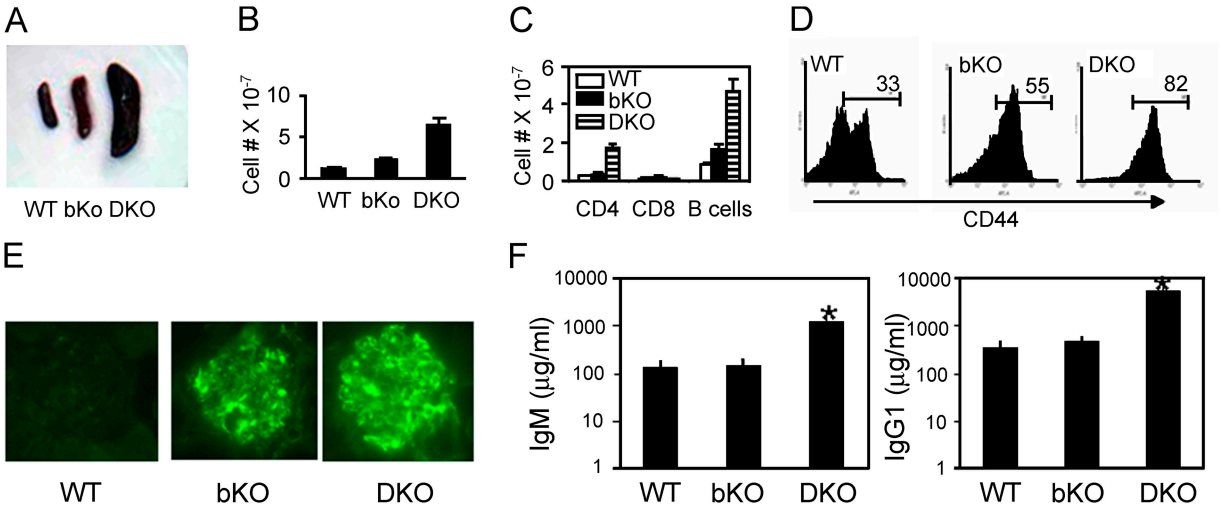

Figure 4. Severe lymphoproliferative disorders in older *Gadd45β*$^{-/-}$ and *Gadd45β*$^{-/-}$ × *Gadd45γ*$^{-/-}$ mice. Female wild-type (WT), *Gadd45β*$^{-/-}$ (bKO), and *Gadd45β*$^{-/-}$ × *Gadd45γ*$^{-/-}$ (DKO, double "knockout") mice were killed when they were ~10–12 mo of age. Spleens were taken from these mice. (A) Splenomegaly. (B) The number of total splenocytes was recorded. (C) Splenocytes were stained with anti-CD4, anti-B220, and anti-CD8 and analyzed by flow cytometry; also, absolute numbers were calculated. (D) Splenocytes were also stained with anti-CD4 and anti-CD44, a marker for effector/memory T cells, and analyzed by flow cytometry. Data are representative for three independent experiments. (E) Detection of IgM deposition in glomeruli by immunofluorescence reveals the presence of IgM-containing immunocomplexes in glomeruli of *Gadd45β*$^{-/-}$ mice and *Gadd45β*$^{-/-}$ × *Gadd45γ*$^{-/-}$ mice. (F) Total IgM and IgG1 production. *, $P < 0.01$, determined by Mann-Whitney statistics.

data), we observed immune complex deposits in renal glomeruli of *Gadd45β*-deficient mice. Levels of total IgM and IgG1 were also increased in the *Gadd45β*×*Gadd45γ* double-deficient mice compared with wild-type mice and *Gadd45β*-deficient mice (Fig. 4 F). Therefore, Gadd45β and Gadd45γ synergistically control peripheral tolerance and prevent systemic autoimmunity.

DISCUSSION

In this work, we extended our understanding of the in vivo role of Gadd45β and Gadd45γ in homeostasis of the immune system and immune tolerance. We have found that Gadd45β and Gadd45γ play a critical role in the resolution of immune responses to prevent autoimmunity. We have shown that Gadd45β (this study) and Gadd45γ (4) are required for AICD in effector Th1 cells. In addition, Gadd45β is required to limit the proliferation of $CD4^+$ T cells in response to TCRs or cytokines. As a result, *Gadd45β* deletion caused a more severe and progressive form of the organ-specific autoimmune disease EAE. Strikingly, we also found that *Gadd45β*×*Gadd45γ* compound deletion resulted in a severe lymphoproliferative disorder and break of tolerance to an autoantigen. Therefore, our data strongly suggest that Gadd45β and Gadd45γ play a critical role as a molecular "double-edged sword" in Th1 type immune responses. Certainly, this role is important in the generation of Th1 cells at the initiation phase of immune responses; however, it is also used later to turn off the immune responses. Loss of such a regulatory mechanism will profoundly compromise both the initiation and the termination of an immune response.

EAE is a murine model of human multiple sclerosis caused primarily by Th1 type autoimmune infiltration of the neuronal tissues such as the brain and spinal cord. Interestingly, deficiency in IFN-γ, a prototypic Th1 cytokine, or IFN-γRa resulted in exacerbated EAE (16). Although we have observed that *Gadd45β* and *Gadd45γ* deletion resulted in reduced Th1 responses in the *Listeria* infection model in vivo and IL-12–driven Th1 differentiation in vitro (3, 4), we think exacerbation of EAE in *Gadd45β*-deficient mice is based on a different mechanism from that in IFN-γ–deficient mice. First, there were large numbers of neutrophils in the CNS of mice lacking IFN-γ or IFN-γRa (14, 15). In

Table IV. Spontaneous development of antibodies to dsDNA and histone in the serum of female wild-type mice and Gadd45β×Gadd45γ double-deficient mice

	Anti-dsDNA (IgM)		Anti-dsDNA (IgG1)		Antihistone (IgM)		Antihistone (IgG1)	
Genotype	WT	DKO	WT	DKO	WT	DKO	WT	DKO
Positive mice	0 ($n = 7$)	4 ($n = 5$)	0 ($n = 7$)	4 ($n = 5$)	0 ($n = 7$)	3 ($n = 5$)	0 ($n = 5$)	4 ($n = 5$)
Average titer	>1:100	1:5,000[a]	>1:100	1:5,000[a]	>1:100	1:29,166[a]	>1:100	1: 22,500[a]

[a]$P < 0.05$, determined by Mann-Whitney statistics.

contrast, we did not observe any neutrophils in the CNS of *Gadd45β*-deficient mice. Second, IFN-γ did not act directly on Th1 cells to affect their proliferation (17, 18). In contrast, Gadd45β directly inhibited the proliferation of Th1 cells. Therefore, Gadd45 family members provide a novel mechanism to suppress Th1 cells.

Besides Gadd45β and Gadd45γ, Gadd45α has been shown to be important for controlling autoimmunity. The lack of Gadd45α in mice resulted in autoimmune symptoms bearing similarities to lupus (19). In contrast with the role of Gadd45β and Gadd45γ in controlling peripheral activated T cells, Gadd45α likely performs its function in the thymus because it is not substantially expressed in peripheral T cells (unpublished data). Gadd45α deletion may alter thymus-mediated central tolerance that leads to autoimmunity. However, because they are highly expressed in peripheral T cells, Gadd45β and Gadd45γ are likely more critical for the peripheral tolerance.

MATERIALS AND METHODS

Mice. *Gadd45β*$^{-/-}$ and *Gadd45γ*$^{-/-}$ mice were generated as described previously (3, 4). *Gadd45β*$^{-/-}$ and *Gadd45γ*$^{-/-}$ mice on 129×C57BL/6 mixed backgrounds were intercrossed to generate *Gadd45β*$^{-/-}$ × *Gadd45γ*$^{-/-}$ double-deficient mice. *Gadd45β*$^{-/-}$ were backcrossed onto C57Bl/6 background for six generations and wild-type and *Gadd45β*$^{-/-}$ littermates were used for in vivo and in vitro experiments.

Culturing Th1 cells. Splenocytes and lymph node cells were stained with anti-CD8 antibody and anti–MHC class II. Then CD8$^+$ cells and B cells were depleted using goat anti–rat Ig and goat anti–mouse Ig (QIAGEN) antibody conjugated magnetic beads. The CD4$^+$ enriched cells were stained with anti-CD62L and anti-CD44 antibodies (GE Healthcare). Naive (CD62L$^+$CD44^{low}) CD4$^+$ T cells were sorted on a Becton Dickinson Vantage. Naive CD4$^+$ T cells were cultured on 24-well plates precoated with 10 μg/ml anti-CD3 (purified in our lab) and 5 μg/ml anti-CD28 (purified in our lab). Th1 conditions were formulated using 3.4 ng/ml IL-12, 20 U/ml human IL-2, and 2 μg/ml anti–IL-4 (clone 11B11) antibody. At 48 h after starting the culture, the cells were replated to another culture dish free of anti-CD3 with freshly added 5 U/ml human IL-2. Cells were cultured further for another 2 d, washed, and stimulated with either plate-bound anti-CD3 or IL-12 (3.4 ng/ml) and 10 ng/ml IL-18 for various times.

AICD assay. Effector Th1 cells were stimulated with plate-bound anti-CD3 for 6–8 h. Cells were harvested and subjected to TUNNEL and annexin V assay. The TUNNEL$^+$ and annexin V$^+$ cells were analyzed using flow cytometry. We used ApopTag Fluorescein Direct in situ Apoptosis Detection Kit (Chemicon) for TUNNEL analysis. Annexin V–PE were obtained from BD Biosciences.

Proliferation assay. For staining with CFSE (Invitrogen), cells at the concentration of 10^7 cells/ml in PBS were incubated at 37°C for 10 min with 0.5 μM CFSE. Next, cells were cultured for 72 h. Cell division were analyzed using flow cytometry.

Adoptive transfer and EAE induction. Splenocytes of donor mice were incubated with anti-CD4 microbeads and CD4$^+$ T cells were purified using MACS columns per manufacturer's instruction (Miltenyi Biotec). The control group consisted of Rag1$^{-/-}$ mice into which CD4$^+$ T cells from wild-type mice had been transferred. The experimental group consisted of Rag1$^{-/-}$ mice into which CD4$^+$ T cells from "knockout" mice had been transferred. 3×10^6 cells were injected in the tail vein of Rag1$^{-/-}$ recipient mice. After 24 h, Rag1$^{-/-}$ recipients were used for EAE induction by subcutaneous injection of 50 μg $MOG_{35–55}$ peptide in incomplete Freund's adjuvant and 50 μg of heat-inactivated *M. tuberculosis*. 200 ng of pertussis toxin was injected intraperitoneally on days 0 and 2. Mice were monitored for clinical signs of EAE, scored as follows: 0, normal; 1, flaccid tail; 2, hind limb weakness or abnormal gait with poor ability to right from supine; 3, partial hind limb paralysis; 4, both hind limbs paralyzed with or without forelimb paralysis and incontinence; 5, moribund state. EAE in wild-type and Gadd45β$^{-/-}$ mice were similarly induced. The experiments were performed in accordance with the regulation of the Institutional Animal Care and Use Committee of University of Pittsburgh and Yale University.

Measurement of antibody production. To measure histone-reacting autoantibodies, the plates were coated with 10 μg/ml calf thymus type-II S histone (Sigma-Aldrich) at 4°C for overnight and blocked with 1% BSA for 1 h at room temperature. Serially diluted serum samples were added and incubated for overnight at 4°C. Plates were washed (0.05% Tween 20 in PBS) and bound Ig was revealed with alkaline phosphatase–conjugated goat anti–mouse IgG1, IgG2a, or IgM (Southern Biotechnology Associates, Inc.), using *p*-nitrophenyl phosphate (Sigma-Aldrich) as a substrate. The plates were read at 405 nm on a microplate reader (Molecular Devices).

To measure dsDNA-reacting autoantibodies, pure DNA was prepared from salmon sperm DNA (Calbiochem-Novabiochem) by phenol/chloroform extraction. Microtiter plates were coated for O/N at 4°C with 50 μg/ml poly-L-lysine (Sigma-Aldrich) in PBS. After washing, plates were coated with 10 μg/ml dsDNA for 2 h at room temperature. Plates were washed twice and blocked with 1% BSA (Sigma-Aldrich) in PBS for 1 h at room temperature. Sera at varying dilutions in PBS/Tween 20 were incubated for overnight at 4°C, and bound antibodies were detected as described before.

CNS cell isolation and ex vivo stimulation. To isolate cells from the CNS, mice were deeply anesthetized and perfused intracardially with RPMI 1640 medium. Brain and spinal cord cell suspensions were incubated with 1 mg/ml collagenase II (Sigma-Aldrich), at 37°C for 20 min, and mononuclear cells were isolated by discontinuous Percoll (GE Healthcare) gradient.

Histological analysis. Mice were deeply anesthetized, perfused intracardially with cold RPMI 1640 and then with 4% paraformaldehyde. Brains and spinal cords were removed and fixed in zinc-buffered formalin. Tissues were processed, sectioned, and stained with hematoxylin and eosin by the Pathology Laboratory at University of Pittsburgh. Kidney specimens embedded in OCT were snap frozen on isopentane/dry ice. 6-μm-thick cryostat sections fixed in 4% paraformaldehyde were stained with Alexa 488–conjugated goat anti–mouse IgM (Invitrogen).

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