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Transgenic Expression of the Viral FLIP MC159 Causes lpr/gld-Like Lymphoproliferation and Autoimmunity

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Death receptor-induced programmed cell death (PCD) is crucial for the maintenance of immune homeostasis. However, interference of downstream death receptor signaling by genetic ablation or transgenic (Tg) expression of different apoptosis inhibitors often impairs lymphocyte activation. The viral FLICE (caspase-8)-like inhibitor proteins (v-FLIPs) are potent inhibitors of death receptor-induced apoptosis and programmed necrosis. We generated Tg mice expressing the v-FLIP MC159 from Molluscum contagiosum virus under the control of the H2Kb class I MHC promoter to examine the role of death receptor-induced PCD in the control of immune functions and homeostasis. We found that expression of MC159 led to lymphoproliferation and autoimmunity as exemplified by T and B lymphocyte expansion, accumulation of TCRαβ+CD3+B220−CD4−CD8− lymphocytes in secondary lymphoid organs, elevated serum Ig levels, and increased anti-dsDNA Ab titers. These phenotypes were caused by defective death receptor-induced apoptosis, but not by defective passive cell death in the absence of mitogenic stimulation. Lymphocyte activation was normal, as demonstrated by normal thymidine incorporation and CSFE dilution of T cells stimulated with anti-CD3 and anti-CD28 Abs. In addition, effector CD8+ T cell responses to acute and memory lymphocytic choriomeningitis virus infections were unaffected in the Tg mice. These phenotypes are reminiscent of the lpr and gld mice, and show that the v-FLIP MC159 is a bona fide PCD inhibitor that does not interfere with other essential lymphocyte functions. Thus, the MC159-Tg mice provide a model to study the effects of PCD in immune responses without hampering other important lymphocyte functions. The Journal of Immunology, 2006, 177: 3814–3820.

Tolerance and prevention of autoimmunity is achieved by diverse mechanisms. Developing T cells in the thymus undergo negative selection to eliminate self-reactive T cells. However, this central tolerance mechanism alone is not sufficient to eliminate all autoreactive T cells. Additional mechanisms in the periphery are required to maintain immune homeostasis and exert further control against autoreactive lymphocytes. Programmed cell death (PCD) within the immune system eliminates unwanted lymphocytes and is vital to the prevention of autoimmunity. The importance of PCD in the control of immune homeostasis is highlighted by mutations in CD95/Fas/APO-1 (lpr) and CD95L/FasL/APO-1-L (gld), which cause lymphoproliferation and autoimmune diseases due to disrupted lymphocyte apoptosis (1). Dominant-interfering mutations in CD95 or CD95L cause a similar autoimmune disease in humans called autoimmune lymphoproliferative syndromes (2). Besides CD95, other members of the TNFR superfamily may also contribute to immunological tolerance by modulating lymphocyte PCD (3).

Apoptosis is the major PCD pathway induced by death receptors. In the case of CD95, ligand binding to the preassembled trimeric receptor triggers binding of the adapter Fas-associated death domain (FADD) via interaction in the death domains (DDs). Subsequent recruitment of caspase-8 and/or caspase-10 through the death effector domains of FADD initiates their proteolytic cleavage and the caspase cascade, which ultimately leads to the apoptotic death of the cell (4). For TNFR-1, binding of an additional adapter TNFR-associated DD to TNFR-1 initiates the formation of a transient membrane-associated complex that also contains receptor-interacting protein and TNFR-associated factor 2, but not FADD or caspase-8 or -10. Upon endocytosis of this membrane complex, TNFR-1 dissociates from the complex and triggers the binding of FADD and caspase-8 or -10 to form a more stable cytoplasmic complex (5). Although the importance of death receptor-induced apoptosis in immune homeostasis is well-established, genetic inhibition of caspase-8 or caspase-10 activity by transgenic (Tg) expression of the baculoviral apoptosis inhibitor p35, the cowpox virus inhibitor CrmA, or the cellular FLIP failed to recapitulate the autoimmune phenotypes of the lpr/gld mice (6–9). Similarly, inhibition of FADD by genetic ablation or a dominant-negative FADD transgene did not result in autoimmunity (10–12).

The lack of development of autoimmune disease in these models may be due to a lack of expression of the transgenes in mature lymphocytes. In addition, FADD regulates lymphocyte cell cycle progression, while caspase-8 has an additional role in mediating TCR-induced NF-κB activation (13–15). Thus, inhibition of FADD or caspase-8 activity hampered lymphocyte development, compromised lymphocyte activation, and led to immunodeficiencies.

Besides apoptosis, death cytokines can induce an alternative form of PCD that we have termed "programmed necrosis" (16, 17). Death cytokine-induced programmed necrosis requires the serine/threonine kinase receptor-interacting protein, but occurs in the absence of caspase activation. In fact, apoptosis is the dominant PCD pathway used by cells, but caspase inhibition facilitates...
programmed necrosis (16, 18). It is interesting to note that TCR-induced PCD appears to trigger both apoptosis and programmed necrosis in T cells, suggesting that, like apoptosis, programmed necrosis may similarly regulate immune homeostasis and contribute to the prevention of autoimmunity (19). Thus, the lack of autoimmune phenotypes in mouse models where apoptosis was inhibited might be due to compensatory effects of programmed necrosis in lymphocytes.

The viral FLIP (v-FLIP) MC159 from *Molluscum contagiosum* virus is a potent inhibitor of both death receptor-induced apoptosis and programmed necrosis (16). In this study, we examined the role of apoptosis and programmed necrosis in the prevention of autoimmunity by Tg expression of MC159-GFP fusion protein under the control of the ubiquitously expressed H2Kb promoter (20). Our data indicate that expression of MC159 specifically blocked apoptosis and programmed necrosis induced by multiple death receptors. The MC159-Tg mice exhibited B and T cell lymphoproliferation, elevated serum Ig levels and increased anti-dsDNA Ab titers in a gene dose-dependent manner. Strikingly, the lymphoproliferation was marked by the accumulation of a unique population of TCRαβ CD3- CD20+ CD4+ CD8- T cells that is characteristic of the lpr/gld mice. Other lymphocyte functions, such as proliferation in response to TCR and BCR stimulation, as well as acute and memory T cell responses to LCMV infection, were unaffected by the MC159 transgene. Hence, these results demonstrated that inhibition of multiple death receptor-induced PCD pathways was sufficient to cause lymphoproliferation and autoimmune-like phenotypes.

**Materials and Methods**

**Generation and analyses of Tg mice**

The DNA fragment containing an in-frame fusion of MC159 to enhanced GFP has been described (16) and was cloned into the H2K-i-LTR vector at the NorI site (20). Linearized DNA was injected into inbred C57Bl/6J embryos. Transgenic founders were screened by PCR genotyping using the following primers: 5′-ggcatggacgagctgtaca-3′ and 5′-ggcttggctttctgtgtc-3′. A total of 11 founders was obtained. Phenotypic analyses of the Tg mice and wild-type (WT) littersmates were performed at 10 wk of age unless otherwise stated. All experimental procedures have been reviewed and approved by the University of Massachusetts Medical School institutional review board. Total RNA was extracted from different tissues using the RNeasy RNA extraction kit (Qiagen) and expression of the transgene in different tissues was determined by RT-PCR using the primers 5′-gcccttggctttctgtgtc-3′ and 5′-gccgttggctttctgtgtc-3′. A total of 11 founders was obtained. Western blot analysis of the Tg mice and wild-type WT littersmates were performed at 10 wk of age unless otherwise stated. All experimental procedures have been reviewed and approved by the University of Massachusetts Medical School institutional review board.

**Cell death assays**

Total splenic T cells, CD4+ T cells, or B cells were purified from 10-wk-old mice using the negative selection kits from Dynal. Purified T cells were stimulated with 5 μg/ml Con A for 2 days. Cells were then washed with 20 mg/ml α-methyl d-nanoppyranoside and PBS, and cultured for 2 days in RPMI 1640 medium supplemented with 100 μM recombinant human IL-2 before testing for response to anti-CD3 or CD95-induced PCD. CD95L (Axxora) and anti-CD95 Ab (clone Jo2) were used at 1 μg/ml for anti-CD95-induced apoptosis, 2 μg/ml protein A was used as cross-linker. Cell death was assessed by annexin V and propidium iodide (PI) staining 6 h after treatment by flow cytometry. Percent cell loss was calculated using the formula: (1 – (number of live cells in treated sample/number of cells in untreated sample)) × 100%.

**Cell proliferation assay**

T cells and B cells were purified using magnetic bead purification (DynaL). Purified T or B cells were cultured at a density of 5 × 10^5 cells/well in a flat-bottom 96-well plate. T cells were stimulated with the indicated doses of plate-bound anti-CD3 Ab. Where indicated, the cells were costimulated with 10 μg/ml anti-CD28 Ab. Two days later, cells were pulsed with 1 μCi [3H]thymidine for 8 h to determine cellular proliferation. For CFSE labeling, purified T cells were resuspended at 2 × 10^5 cells/ml in PBS. A total of 1 μM CFSE was added to the cells and incubated at 37°C for 15 min. Cells were stimulated with 10 μg/ml anti-CD3 and 10 μg/ml anti-CD28 Abs. Dilution of CFSE on days 2 and 3 poststimulation was determined by flow cytometry using a BD Biosciences LSRII cytometer.

**Viruses**

LCMV, strain Armstrong, and Pichinde virus (PV), strain AN3739 stocks were prepared in baby hamster kidney cells (BHK21) as previously described (21). Primary virus-specific T cell responses were measured 8 days after i.p. infection with 5 × 10^5 PFU of LCMV, and mice were considered immune 6 wk after infection. Recall T cell responses were measured 6 days after infection of LCMV-immune mice with 1 × 10^5 PFU of LCMV or with 1 × 10^5 PFU of PV.

**Intracellular cytokine staining**

Viruses-specific CD8+ T cells were quantified by intracellular detection of IFN-γ using the Cytofix/Cytoperm kit (Pall Corp). CFSE-labeled T cells were cultured at a density of 5 × 10^5 cells/ml in RPMI 1640 medium supplemented with 100 U/ml recombinant human IL-2 (BD Pharmingen), and 1 μg/ml GolgiPlug at 37°C/5% CO2 for 5 h. PBLs were stained with Abs specific for CD8 and CD44, fixed, and permeabilized with Cytofix/Cytoperm solution and stained with Ab specific for IFN-γ (XMG1.2; BD Biosciences). The samples were analyzed using the LSRII flow cytometer (BD Biosciences).

**Determination of serum Ig levels and anti-dsDNA Ab titers**

Plates were coated overnight at 4°C with 1 μg/ml isotype-specific goat anti-mouse Ig (Caltag Laboratories) in PBS, washed with PBS containing 0.1% Triton X-100, and blocked for 1 h at room temperature with 10% normal goat or mouse serum. Serum dilutions of 1/5, 1/15, 1/45, and 1/135,000 in a serum dilution buffer were added and incubated at room temperature for 2 h. After washing, plates were incubated with HRP-conjugated goat anti-mouse IgG or IgM (no. M30807; Caltag Laboratories) for 90 min. After washing, plates were developed in CA buffer (0.0233 M citric acid, 0.0534 M Na2HPO4, 1.6 mg/ml BSA, and 0.1% Triton X-100, and blocked for 1 h at room temperature with 10% normal goat or mouse serum). The samples were analyzed using the LSRII flow cytometer (BD Biosciences).

**Results**

Expression of the MC159 transgene in multiple tissues

We generated Tg mice expressing the PCD inhibitor MC159 under the control of the class I MHC H2Kb promoter (20). To facilitate detection of transgene expression, MC159 was fused at the C terminus to the GFP. Eleven independent founders were obtained with variable expression in different tissues. Expression of the transgene at miRNA and protein levels was confirmed by RT-PCR and Western blot. Line 13, which exhibited the strongest expression of the transgene in multiple tissues, was chosen for further analysis (Fig. 1A). We confirmed the expression of the MC159-GFP fusion protein in the liver, lung, and lymph nodes by Western blot analysis (Fig. 1B). Moreover, the expression of the transgene in different lymphocyte populations such as B cells and T cells was further confirmed by flow cytometry using GFP fluorescence as an indicator (Fig. 1C).
MC159 protects against death receptor-induced apoptosis and programmed necrosis

The v-FLIP MC159 is a potent inhibitor of death receptor-induced apoptosis and programmed necrosis (16). We found that Tg B and T cells were protected from CD95-induced apoptosis, although the protection was partial (Fig. 2A). Moreover, activated Tg CD4+ T cells were resistant to low dose (1 μg/ml) anti-CD3-induced PCD, which involved caspase-mediated apoptosis and caspase-independent programmed necrosis (Fig. 2B) (19). Again, the protection conferred by the MC159 transgene was partial, because PCD induced by a high dose of anti-CD3 Ab (10 μg/ml) was not protected by the transgene (Fig. 2B). Furthermore, Tg tail skin fibroblasts were resistant to TNF-induced apoptosis and programmed necrosis (data not shown). The protection conferred by the transgene was specific to death receptor-induced cell death, because the Tg lymphocytes were not protected against staurosporine-induced apoptosis (data not shown) or the Bcl-2-regulated spontaneous lymphocyte death in the absence of external stimuli (Fig. 2C).

Normal lymphocyte development and lymphoproliferation in the MC159 Tg mice

Transgenic expression of the v-FLIP E8 and c-FLIPs (long form) increased spontaneous apoptosis of developing thymocytes and reduced thymic cellularity (9, 24). By contrast, c-FLIPs (short form) and K13 v-FLIP Tg mice do not exhibit any abnormalities in thymic development (8, 25). We found that thymic development as determined by the percentages of CD4-8 T, CD4-8 T, and CD4-8 SP T cells, but not in the more immature DN or DP thymocytes (data not shown). By contrast, examination of peripheral lymphoid organs revealed moderate splenomegaly and lymphadenopathy as exemplified by a 1.5- to 2-fold increase in splenic cellularity (Fig. 4A). The lymphoproliferation was accompanied by a skewed B/T ratio in the Tg mice compared with WT littermate controls (Fig. 4B). For instance, the percentage of B220+ B cells in the mesenteric lymph nodes increased from 33.2% in WT mice to 57.7% in Tg mice, while the percentage of T cells decreased from 64.4% in the WT to 38.8% in the Tg mice (Fig. 4B). Similar skewing of B-T ratio was observed in the spleen, inginal, axillary, and brachial lymph nodes (data not shown). The skewed B-T ratio was not caused by defective T cell homeostasis, because the number of T cells was comparable in WT and Tg littermates (Fig. 4C) and that the ratio of CD4+ to CD8+ T cells was the same between WT and Tg littermates (Fig. 4D). Rather, the mild lymphoproliferation was caused by an expansion of B cells (Fig. 4E). Flow cytometric analysis of the different B cell subsets revealed that B cell development in the bone marrow was normal (data not shown) and that the expansion was caused by an accumulation of mature follicular B cells (Fig. 4F). The accumulation of B cells in the Tg
were calculated using the Student t test. B, Lymphocytes from mesenteric lymph nodes were analyzed for cell surface expression of B220 and CD3. C, Number of CD3\(^+\)B220\(^-\) T cells in the spleen of WT (n = 7) and Tg (n = 8) mice was determined by flow cytometry. D, Splenic CD3\(^+\)B220\(^-\) T cells were gated and examined for expression of CD4 and CD8. E, Number of B220\(^-\)CD3\(^+\) B cells in the spleen of WT (n = 7) and Tg (n = 8) mice was determined by flow cytometry. F, Different B cell populations in the spleen of WT (n = 5) and Tg mice (n = 4) were identified by flow cytometry. The different B cell populations were defined as follows: B1 B cells: IgM\(^+\)CD21\(^+\)CD23\(^+\)CD5\(^+\)CD43\(^-\); marginal zone B cells: IgM\(^+\)CD21\(^-\)CD23\(^+\)CD24\(^+\)CD38\(^-\); follicular B cells: IgM\(^+\)IgD\(^+\)CD21\(^-\)CD23\(^-\); T1 B cells: IgM\(^+\)CD21\(^-\)CD23\(^+\)CD24\(^+\)CD38\(^-\); and T2 B cells: IgM\(^+\)CD23\(^+\)CD24\(^-\)CD38\(^-\). The p values were calculated using the Student t test.

Normal lymphocyte activation and response to LCMV infections in MC159-Tg mice

The K13 v-FLIP Tg lymphocytes exhibited hyperproliferative response to mitogenic stimulation due to enhanced NF-κB activation (25). Thus, the B cell expansion in the MC159-Tg mice may be caused by uncontrolled lymphocyte activation. We therefore examined B and T cell responses to various mitogenic stimuli. We found that B cell proliferation induced by CpG oligonucleotides, LPS, or anti-IgM Ab was normal in Tg B cells (Fig. 5A). Similarly, T cell proliferation in response to anti-CD3 and anti-CD28 stimulation was unaffected by the MC159 transgene (Fig. 5B). Because the resistance of Tg lymphocytes to PCD induction could have biased the interpretation of the thymidine uptake experiment, we labeled Tg and WT T cells with CFSE and examined CFSE dilution upon stimulation anti-CD3 and anti-CD28 Abs to more definitively evaluate the proliferative potential of the Tg lymphocytes. We found that the proliferation of Tg T cells was equivalent to that of WT T cells. In fact, T cells from Tg/Tg mice, which express higher amount of the MC159-GFP fusion protein (Fig. 7 below), proliferated equally well compared with WT cells (Fig. 5C). Moreover, analyses of cell cycle progression by PI staining of DNA and expression of CD25 and CD69 in response to anti-CD3 and anti-CD28 stimulation revealed no difference between Tg and WT T cells (data not shown). These data therefore indicate that lymphocyte responses to mitogenic stimulation were normal and unlikely to contribute to the lymphoproliferation in the Tg mice.

The normal proliferative responses of the MC159-Tg lymphocytes contrast the reported results of a recently described T cell-specific MC159-Tg mouse line that exhibited defective CD8\(^+\) T cell responses to viral infections and postactivation survival defect (26). To more definitively evaluate CD8\(^+\) T cell responses in our Tg mice, we tested our MC159-GFP-Tg mice for acute and memory T cell responses to LCMV. We found that the frequencies of LCMV peptide-specific effector CD8\(^+\) T cells in the MC159-Tg mice were comparable to that of the WT controls (Fig. 6A). Moreover, CD8\(^+\) T cell recall responses to LCMV infection were normal in the Tg mice (Fig. 6B). Furthermore, LCMV-immune mice mounted normal CD8\(^+\) T cell responses to infection with the heterologous virus PV (data not shown). Collectively, these data indicate that Tg expression of MC159 does not compromise lymphocyte responses to antigenic stimulation.
IFN-CD8. Tg/Tg mice were analyzed for expression of CD4 and mice per group. Results shown are representative of at least six days later, PBLs were analyzed for production of IFN-

TCR characteristic of the lpr cells (CD3

sent percentages of cells in each quadrant. B220 expression were gated and analyzed for expression of CD44 and IFN-γ. B, LCMV-immune mice were rechallenged with LCMV. Seven days later, PBLs were analyzed for production of IFN-γ as described in Materials and Methods. Results shown are representative of at least six mice per group.

Development of autoimmune disease in MC159-Tg mice

It was surprising that the MC159-Tg mice exhibited mild B cell lymphoproliferation without manifestation of other symptoms associated with lpr/gld-like autoimmune disease. We reasoned that the lack of autoimmune phenotype could be attributed to the partial protection of death receptor-induced PCD (Fig. 2). We therefore determined whether increasing the expression of the transgene might further exacerbate lymphoproliferation and cause autoimmunity. To this end, we intercrossed the MC159-Tg mice to obtain mice containing twice the copy number of the transgenes (Tg/Tg as opposed to Tg/WT). Homozygosity of the Tg/Tg mice was confirmed by real-time PCR using genomic DNA as templates (data not shown). Flow cytometric analyses of GFP expression in B cells, T cells, dendritic cells (DCs), and macrophages revealed enhanced expression of the transgene as determined by GFP fluorescence (Fig. 7, A and B). Strikingly, flow cytometric analysis revealed the expansion of a population of T cells that were CD3+ B220+ in the Tg/Tg mice (Fig. 7C). Unlike CD3+ B220+ T cells, which were either CD4+ or CD8+, the CD3+ B220+ T cells expressed neither CD4 nor CD8 (Fig. 7D). Moreover, these double-positive cells were of the αβ T cell lineage (Fig. 7E). Thus, the cellular phenotypes of the Tg/Tg mice resembled that of the lpr/gld mice. The presence of the TCRαβ+ CD3+ B220+ CD4− CD8− T cells, a unique population characteristic of the lpr/gld mutation. A, GFP fluorescence of WT/WT (gray curve), Tg/WT, and Tg/Tg B cells (CD3+ B220+) and T cells (CD3+ B220+). The numbers in parentheses represent the geometric mean of GFP fluorescence. B, GFP fluorescence of splenic DCs and macrophages in WT/WT (gray curve), Tg/WT, and Tg/Tg mice. The numbers in parentheses represent the geometric means of the GFP fluorescence. C, Splenocytes from 10-wk (top) and 5-mo-old (bottom) Tg/Tg mice and WT controls were analyzed for expression of B220 and CD3 on the cell surface. The numbers represent percentages of cells in each quadrant. D, The CD3+ B220+ and the CD3+ B220− cells from 5-mo-old Tg/Tg mice were analyzed for expression of CD4 and CD8. E, Splenic CD3+ B220+ cells from Tg/Tg mice were gated and analyzed for expression of the TCRβ or TCRγδ. F, Splenocytes that were negative for CD3 and B220 expression were gated and analyzed for expression of CD11c. Results were representative of three mice per group.
Tg/WT or Tg/Tg mice (Fig. 7F). Thus, the lymphoproliferation did not appear to be driven by an increase in DC number.

Elevated serum Ig levels are hallmarks of lpr/gld-like autoimmune disease. We therefore examined serum Ig levels and found that the Tg/Tg mice exhibited elevated levels of all serum Ig iso-types tested (Fig. 8, A–E). In contrast, the serum Ig levels of Tg/WT mice were mostly normal, although IgG2b level was slightly elevated compared with age-matched WT controls (Fig. 8D). Furthermore, serum anti-dsDNA Ab titers were elevated in the Tg/Tg mice compared with WT controls (Fig. 8F). Significantly, serum Ig levels of Tg line 43 (Tg43), which exhibits a similar tissue expression pattern as line 13 but lacks expression of MC159 in the lymphoid compartment, were normal compared with WT controls (Fig. 8, A–E). Collectively, these data indicate that inhibition of death receptor-induced apoptosis and programmed necrosis triggers the onset of lpr/gld-like lymphoproliferation and autoimmunity in the MC159-Tg mice without compromising other lymphocyte functions.

Discussion
In this study, we showed that ubiquitous expression of MC159 causes lpr/gld-like autoimmune phenotypes in a gene dose-dependent manner. Our Tg/WT mice developed a mild form of lymphoproliferation that is characterized by expansion of mature follicular B cells. Because MC159 was expressed at similar levels in B and T cells and that Fas-induced apoptosis was equally affected in both populations, our data imply that B cells are more dependent than T cells for proper homeostatic regulation through death receptor-induced PCD. Unlike Tg/WT mice, Tg/Tg mice exhibited B and T cell hyperplasia, elevated serum Ig levels, and increased titers of anti-dsDNA Abs. The lymphoproliferation in the Tg/Tg mice was marked by the appearance of T cells that were TCRαβ+CD3−B220−CD4−CD8−, a signature population in the autoimmune lpr or gld mice. Specific inhibition of apoptosis in DCs by Tg expression of the baculovirus p35 has recently been shown to result in autoimmune disease development (28). However, the p35-DC-Tg mice did not accumulate TCRαβ+CD3−B220−CD4−CD8− cells in peripheral lymphoid organs. Thus, although our transgene was expressed in DCs, the lymphoproliferation in Tg/Tg mice did not appear to be caused by defective DC apoptosis. Because we only observed the lpr/gld-like phenotypes in one founder line, it is possible that the phenotypes were caused by inactivation of an unknown gene in the Tg/Tg mice. However, we reason that the similarity of the phenotypes to the lpr/gld mice argues that the defect was caused by inhibition of death receptor-induced apoptosis and programmed necrosis rather than random inactivation of an unknown gene. Thus, the lack of autoimmune phenotypes in the other founder lines might be due to the weaker expression of the transgene. We are currently intercrossing mice from the other founder lines to determine whether increasing expression of the transgene in the other lines will similarly lead to autoimmune disease development.

Our results are in contrast to those reported by Wu et al. (26), who observed no lymphoproliferation or autoimmune disease in T cell-specific MC159-Tg mice. The different phenotypes can be explained by a requirement to inhibit cell death in both B and T cells for the development of autoimmunity. In fact, tissue-specific inactivation of Fas revealed that Fas ablation in both lymphoid and nonlymphoid cells was required for development of autoimmune disease (29). In agreement with this result, other founder lines that lack expression in lymphocytes did not exhibit any lymphoproliferation or elevated serum Ig levels (Fig. 8). Besides differences in autoimmune disease development, our Tg mice also differ from the T cell-specific MC159 Tg mice in their responses to viral infections. Wu et al. (30) reported that the T cell-specific MC159-Tg mice exhibited defective responses to infections with LCMV, influenza, and the parasite Trypanosoma cruzi. The defective immune responses were attributed to defective postactivation survival and IFN-γ production by CD8+ T cells (30). By contrast, our MC159-Tg mice exhibited normal acute and memory CD8+ T cell responses to LCMV infections. Moreover, we found that LCMV-immune Tg mice elicited normal CD8+ T cell responses when challenged with a heterologous virus PV (data not shown). Our results are consistent with the normal lymphocyte responses to LCMV infections in Fas-deficient lpr mice (31). The disparate phenotypes between the two different Tg lines might be due to differences in the expression level and pattern of the transgene.

The FLIP molecules are thought to regulate apoptosis by binding to and inhibiting the function of FADD and/or caspase-8. Genetic ablation of FADD or caspase-8 revealed a role for FADD in lymphocyte cell cycle progression. Similarly, caspase-8 regulates lymphocyte activation and proliferation through TCR-mediated

### Table 1. Expansion of CD3+ B220+ T cells in 5-mo-old Tg/Tg mice

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<th>WT (×5)</th>
<th>Tg/Tg (×3)</th>
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<td>B220+CD3+ B Cells</td>
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<td>(p = 0.001)</td>
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<td>(1.03 ± 0.18) × 10^7</td>
<td>(5.69 ± 1.97) × 10^7</td>
<td>(4.36 ± 0.50) × 10^7</td>
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Values of *p* were calculated using the Student *t* test.

**FIGURE 8.** Elevated serum Ig levels in MC159-Tg mice. Serum levels of A, IgM, B, IgG1, C, IgG2a, D, IgG2b, and E, IgG3 were determined by ELISA as described in Materials and Methods. F, Serum titers of anti-dsDNA Ab. All serum samples tested were from mice 5–14 mo old. Tg/WT: *n* = 3, Tg/Tg: *n* = 6, WT: *n* = 3, and Tg43: *n* = 3.
NF-κB induction (12, 15, 32, 33). As such, Tg expression of inhibitors of FADD or caspase-8 often interferes with lymphocyte activation. This is in fact the case for c-FLIP: (9, 34). However, it is noteworthy that no overt lymphocyte activation defects were observed in our MC159-Tg mice and the E8 v-FLIP-Tg mice (24). The K13 Tg lymphocytes did exhibit an enhanced response to mitogen stimuli. However, this is likely due to K13-induced constitutive NF-κB activation that is independent of direct effects on TCR signaling (25). Mutational analyses of MC159 indicated that binding to FADD or caspase-8 is not sufficient to mediate its antiapoptotic function (35). Consistent with these data, the recent crystal structures of MC159 show that MC159 does not preclude binding of FADD and caspase-8 to the death-inducing signal complex (36, 37). Similarly, because inhibition of caspase-8 is a prerequisite to initiate programmed necrosis, MC159 does not inhibit programmed necrosis by targeting FADD or caspase-8 (16, 17). These data strongly suggest that MC159 may target other components of the PCD pathway, such as the TNFR-associated factors (38). Because the MC159 transgene is expressed in multiple tissues and it does not interfere with lymphocyte functions, the MC159-Tg mice provide an ideal model to study the effects of PCD on the regulation of immune responses.

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Disclosures

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