

Mutation of E2F2 in Mice Causes Enhanced T Lymphocyte Proliferation, Leading to the Development of Autoimmunity

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Summary

E2Fs are important regulators of proliferation, differentiation, and apoptosis. Here we characterize the phenotype of mice deficient in E2F2. We show that E2F2 is required for immunologic self-tolerance. E2F2^{-/-} mice develop late-onset autoimmune features, characterized by widespread inflammatory infiltrates, glomerular immunocomplex deposition, and anti-nuclear antibodies. E2F2-deficient T lymphocytes exhibit enhanced TCR-stimulated proliferation and a lower activation threshold, leading to the accumulation of a population of autoreactive effector/memory T lymphocytes, which appear to be responsible for causing autoimmunity in E2F2-deficient mice. Finally, we provide support for a model to explain E2F2's unexpected role as a suppressor of T lymphocyte proliferation. Rather than functioning as a transcriptional activator, E2F2 appears to function as a transcriptional repressor of genes required for normal S phase entry, particularly *E2F1*.

Introduction

The decision by a cell to choose between entry into the cell cycle to proliferate, to remain quiescent, or to die is crucial to an organism. Dysregulation of this decision can result in a failure of important reparative, inflammatory, or other adaptive responses, or the aberrant proliferation characteristic of cancer and autoimmune diseases. Cell cycle entry is primarily regulated in late G1 at the restriction point. A number of biochemical events occur coincident with the restriction point which have been linked together in a model for cell cycle control. Activation of cyclin/cdk kinases results in phosphorylation of Rb, causing its dissociation from E2F. Free E2F then activates the expression of DNA synthesis- and G1/S-regulatory genes (Weinberg, 1995). In addition to preventing E2F-mediated transcriptional activation, Rb can also actively repress transcription from promoters carrying E2F binding sites by binding to DNA-bound E2F and recruiting the histone deacetylase HDAC1 (Brehm and Kouzarides, 1999).

The E2F gene family includes at least seven distinct proteins that bind DNA as heterodimers with members of the DP protein family (Nevins, 1998; Dyson, 1998; Helin, 1998; Macleod, 1999). Among the E2F family members, certain biochemical and functional differences have been reported, though it is not yet clear whether the E2F proteins execute distinct functions in normal cells and to what degree their functions overlap (Nevins, 1998). The E2F family of proteins can be divided into three different subgroups based on sequence similarity as well as functional roles. E2Fs 1, 2, and 3 are related by sequence, are similarly regulated by mitogens, bind almost exclusively to Rb, and share the capacity to induce efficient S phase entry in quiescent cells when overexpressed (Johnson et al., 1993; Lukas et al., 1996; DeGregori et al., 1997; Sardet et al., 1997). On the other hand, a second subgroup of E2Fs, E2F4 and E2F5, are not regulated by cell growth, are poor transcriptional activators, and only weakly induce S phase (Müller et al., 1997; Verona et al., 1997), suggesting that they are repressors of E2F-responsive genes (Dyson, 1998; Helin, 1998). An alternate version of E2F3, named E2F3b, may also be included in this subgroup (Leone et al., 2000; He et al., 2000). The third subgroup of E2F proteins includes E2F6, which lacks an activation domain and has been shown to compete for binding to E2F promoter sites and thereby repress transcription (Dyson, 1998; Helin, 1998; Macleod, 1999).

The generation of mouse strains carrying targeted mutations for individual E2F family members has been crucial to elucidate the function of these proteins in vivo. These mouse models confirm that individual members of the E2F subgroups have very different biological properties that do not merely reflect their pattern of expression. However, it is unclear how these differences relate to the target specificity of the different E2Fs in vivo. Mutation of genes included in the first subgroup of E2F proteins, E2F1 and E2F3, result in mice with defects in cell cycle regulation. Notably, E2F3 is required

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for the normal cell cycle-dependent activation of numerous E2F-responsive genes of both primary and tumor cells. E2F3-deficient mice demonstrate that E2F3 is particularly crucial for normal fibroblast proliferation and results in an embryonic lethal phenotype (Humbert et al., 2000). Mutation of E2F1 reveals that it normally functions as a promoter of apoptosis, particularly in developing thymocytes, and as a tumor suppressor possibly via its role as a promoter of apoptosis (Field et al., 1996; Yamasaki et al., 1996, 1998).

The third member of this E2F subgroup, E2F2, was originally identified by low-stringency hybridization as an E2F1-related gene, together with E2F3 (Kaelin et al., 1992; Ivey-Hoyle et al., 1993), and it shows high sequence homology with E2F1 and E2F3. Similar to E2F1 and E2F3, ectopic expression of E2F2 promotes S phase entry in quiescent immortalized rat fibroblasts through the activation of target genes important at the G1/S transition (Lukas et al., 1996; DeGregori et al., 1997). Other experiments have shown that E2F2 has oncogenic activity (Wu and Levine, 1994). Furthermore, E2F2 gene activity is induced during a proliferative response, leading to an accumulation of E2F2 coincident with S phase entry (Ikeda et al., 1996). These results suggest that E2F2 may be necessary at the G1/S transition to promote cell cycle progression. However, recent studies indicate that E2F2 can also trigger apoptosis under certain conditions (Vigo et al., 1999). It is presently unknown whether E2F2 plays either or both functions *in vivo*. Furthermore, although many transcriptional targets are common to E2F1, E2F2, and E2F3, target specificity has also been reported for each gene (DeGregori et al., 1997; Vigo et al., 1999; Müller et al., 2001). The different target specificity suggests that E2F2 could play a unique role *in vivo* that is not shared by other members of the E2F family.

To assess the function of E2F2 in normal growth and development and to test the role of E2F2 in the regulation of proliferation, apoptosis, and/or differentiation *in vivo*, we have inactivated the E2F2 locus in mice by homologous recombination. Mice lacking E2F2 are viable, yet they exhibit defects in T lymphocyte homeostasis leading to a lupus-like autoimmune disorder. E2F2-deficient peripheral T lymphocytes exhibit enhanced proliferation upon TCR-mediated stimulation, as well as a lower threshold for activation. Thus, E2F2 functions *in vivo* as a negative regulator of the immune response by suppressing cellular proliferation of activated lymphocytes.

Results

Targeted Mutation of the E2F2 Gene in Mice

To study the function of E2F2, we generated mice deficient in E2F2 by gene targeting, using the strategy depicted in Figure 1 and Experimental Procedures. One ES cell line produced high percentage chimeras and was used to produce mice heterozygous for the E2F2 mutation. Heterozygous mice carrying a null E2F2 allele were maintained in a mixed (C57Bl/6 \times 129/Sv) genetic background in all experiments described here. The heterozygotes appeared completely normal in their health and appearance and behaved similarly to wild-type in all assays described below (data not shown). When E2F2 heterozygotes (E2F2^{+/-}) were intercrossed, animals of

the E2F2^{-/-} genotype (Figure 1C) appeared at a frequency close to the expected Mendelian frequency: 97 were +/+, 172 were +/- and 82 were -/- ($\chi^2 = 1.42 < < 5.99$, $p = 0.05$), indicating that all genotypes were equally viable. The E2F2^{-/-} mice survived to adulthood, were fertile, and produced normal offspring. We confirmed by Northern analysis that the targeted mutation in the E2F2 gene abolishes its expression in these animals (Figure 1D).

Aged E2F2-Deficient Mice Die of Systemic Autoimmune Disease

Both the gross and microscopic morphology of organs from 4- to 8-week-old E2F2^{-/-} mice were normal (data not shown). However, by 15 months of age 67% of the wild-type animals were alive and apparently healthy, whereas only 27% of the E2F2-deficient animals had survived to this age. To better ascertain the age-dependent abnormalities in the E2F2-deficient mice, four E2F2-deficient and five wild-type littermates were sacrificed at 15 months of age. Remarkably, all of the E2F2-deficient mice, but none of the wild-type littermates, exhibited features of marked autoimmune disease.

All of the aged E2F2-deficient mice displayed gross splenomegaly, with spleen size two to five times higher than that in wild-type littermates. Spleen histology demonstrated white pulp hyperplasia and increased sinusoidal cellularity (Figures 2A and 2B), characteristic of immune hyperreactivity. Several different organs from elderly E2F2-deficient mice accumulated inflammatory infiltrates, although the organs from age-matched wild-type control mice did not. Infiltrates of mononuclear cells were consistently observed in the lungs of elderly E2F2-deficient mice (Figure 2C). Likewise, the livers of elderly E2F2-deficient mice exhibited numerous perivascular infiltrates (Figure 2D). The aged E2F2-deficient mice also demonstrated evidence of skin abnormalities consisting of considerable hair loss, skin wounds, and erythema affecting the head and neck region, reminiscent of some autoimmune/inflammatory diseases (Figure 2F). The observed skin changes were not found in any of the wild-type control mice.

Histology of the kidneys of elderly E2F2-deficient mice demonstrated a membranoproliferative glomerulonephritis that was focal and of moderate intensity. The affected glomeruli were enlarged with a thickened basement membrane and contained perivascular aggregates of inflammatory infiltrates. Immunocomplex deposition was detected in the affected areas, as determined by immunohistochemical analysis with antibodies to mouse IgG (Figure 2E). As shown, the E2F2-deficient kidneys contain prominent immune complex deposition, in sharp contrast to the wild-type kidneys, which do not contain these complexes.

We also assayed serum from elderly wild-type and E2F2-deficient mice for the presence in the serum of anti-dsDNA antibodies. Notably, the titer of IgG antibody against dsDNA observed in the E2F2-deficient mice correlated with the severity of the autoimmune organ damage observed in those mice and was significantly higher than in wild-type controls (relative OD values for E2F2^{-/-} mice, 4.2, 1.9, and 0.3, versus relative OD values for wild-type control mice, <0.3 in all cases; relative OD value of MRL-*lpr* positive control serum, 4.6).

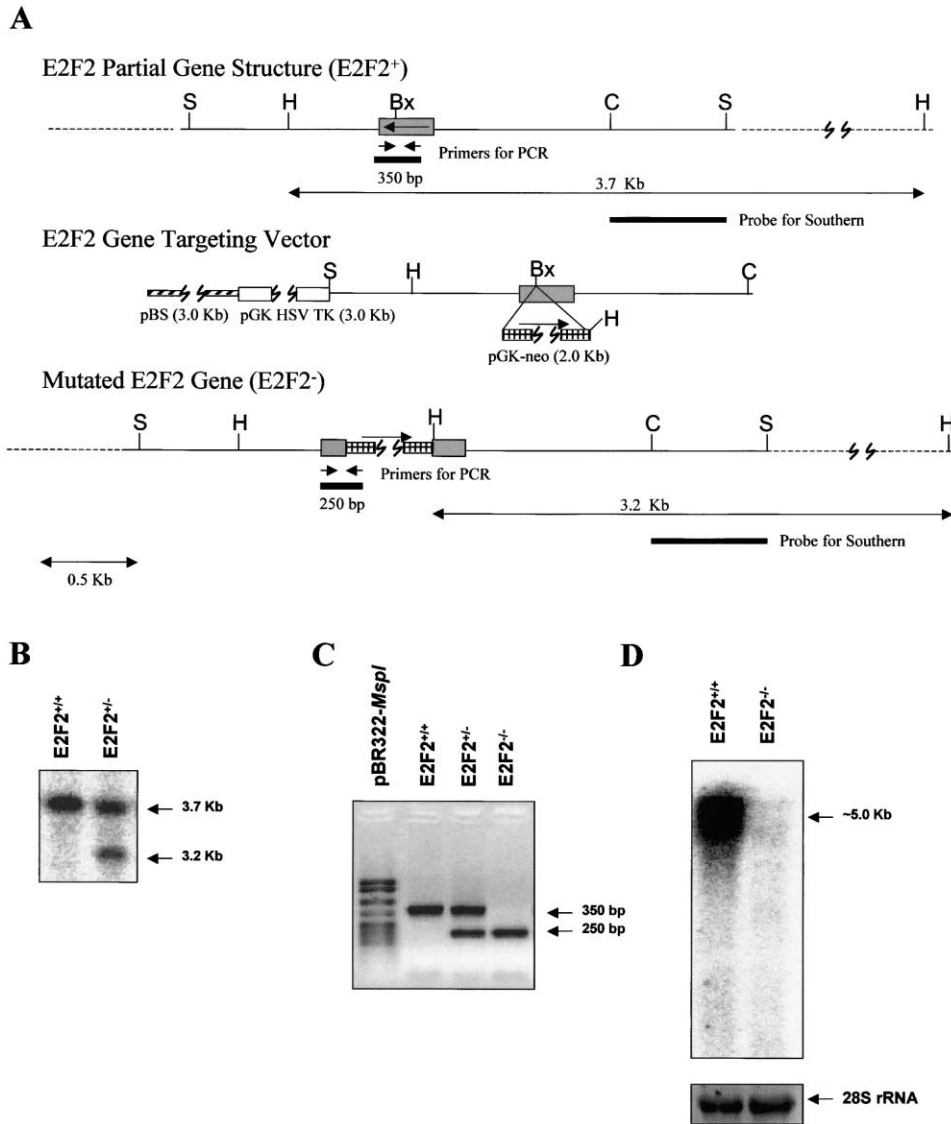


Figure 1. Mutation of the E2F2 Gene

(A) Map of exon 3 (box) and surrounding intronic sequences of mouse E2F2. Restriction enzyme recognition sites are abbreviated as follows: S, SacI; H, HindIII; Bx, BstXI; and C, ClaI.

(B) Southern hybridization using an E2F2 genomic probe (as depicted in [A]) demonstrates the presence of the targeted mutation in ES cell lines. The use of a probe for neo sequences indicated that additional copies of the gene targeting construct do not exist elsewhere in the genome (data not shown).

(C) A genomic PCR was performed to genotype all progeny. PCR primer positions are indicated in (A).

(D) Northern hybridization showing ≈ 5.0 kb band in $+/+$ thymocytes corresponding to the E2F2 mRNA, which is absent in the E2F2^{-/-} thymocytes. The integrity and amount of RNA in each lane was demonstrated by visualizing the 28S RNA band after ethidium bromide staining.

Taken together, the pathological features observed in aged E2F2-deficient mice resemble features found in human and murine autoimmune syndromes. The development of an autoimmune syndrome in E2F2-deficient mice suggests that E2F2 plays a normal role in vivo in the maintenance of immunologic tolerance.

Accentuated Autoimmunity in Male E2F2-Deficient Mice Expressing the Anti-H-Y Transgene

E2F1 is required for normal thymic negative selection (Zhu et al., 1999; García et al., 2000). The autoimmune

syndrome observed in E2F2-deficient mice prompted us to consider if E2F2 is also required for normal thymic negative selection. Thymuses from E2F2^{-/-} mice were found to contain a higher fraction of mature thymocytes (CD4⁺ or CD8⁺) than thymuses from wild-type littermates ($p < 0.001$), especially of the CD8⁺ subset (Figure 3A), with a concomitant decrease in immature double-positive (DP) CD4⁺/CD8⁺ thymocytes. To test whether E2F2 is required for normal thymic negative selection (central tolerance), we introduced the E2F2 mutation into a more informative genetic background:

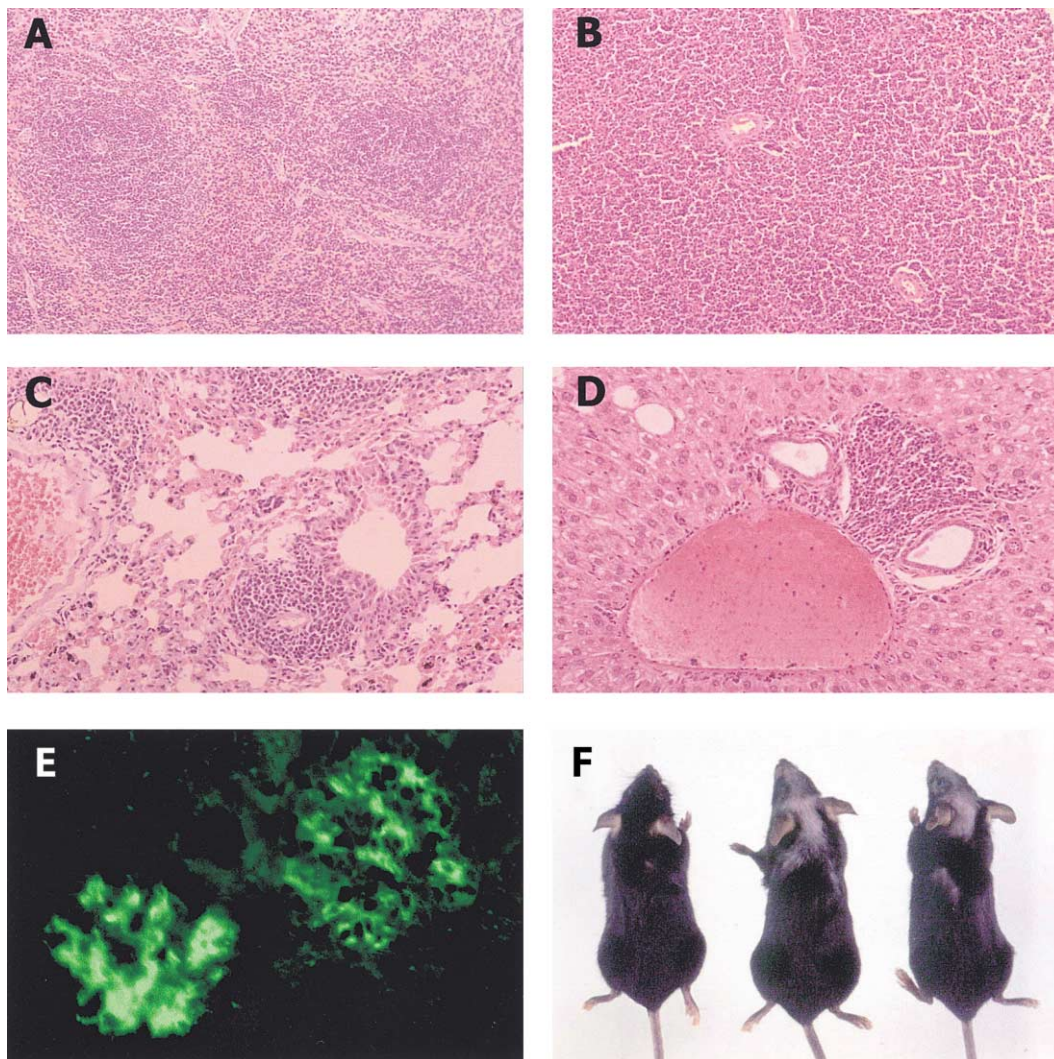


Figure 2. Features of Autoimmune Disease in Older E2F2-Deficient Mice

Organs from wild-type and E2F2^{-/-} mice 15 months of age were harvested and processed as described in Experimental Procedures.

(A and B) Photomicrographs of spleens ($\times 40$) stained with hematoxylin-eosin (H-E). Note the hyperreactive white pulp and the effacement of the normal spleen histology in E2F2^{-/-} mice (B), compared to the normal spleen histology of a wild-type mouse (A).

(C) Lung ($\times 50$) from an E2F2^{-/-} mouse stained with H-E showing inflammatory infiltrates surrounding vessels and bronchioli.

(D) Liver ($\times 50$) from an E2F2^{-/-} mouse stained with H-E showing perivascular infiltrates of mononuclear cells.

(E) Typical IgG deposits in glomeruli of E2F2^{-/-} kidneys ($\times 150$) as shown after staining kidney cryosections with anti-IgG-FITC monoclonal antibody (mAb).

(F) Skin lesions in E2F2^{-/-} mice (center and right), compared to age-matched E2F2^{+/+} mouse (left).

a mouse line carrying a transgene for a T cell receptor specific for male antigen, H-Y (Kisielow et al., 1988). In E2F2^{-/-} Tg⁺ male mice, cell number in the thymus was reduced similarly to E2F2^{+/+} Tg⁺ mice. Likewise, the proportion of CD4⁺/CD8⁺ thymocytes was severely reduced, again similar to wild-type (Figure 3B). Thus, we conclude that mutation of E2F2 does not impair thymic negative selection.

Although thymocyte development appeared normal in E2F2-deficient Tg⁺ male mice, these mice became increasingly sick by 4 months of age and died by 5–7 months of age. By contrast, control E2F2^{+/+} Tg⁺ male mice remained healthy appearing to at least 12 months of age. A detailed analysis of 5-month-old male mice revealed a number of features consistent with an inflam-

matory/autoimmune disorder similar to those seen in 15-month-old E2F2-deficient mice, although more severe, affecting mainly the spleen, liver, and skin (data not shown). By contrast, no overt signs of inflammatory disease were detected in Tg⁺/E2F2^{+/+} male mice or Tg⁺/E2F1^{-/-} male mice (García et al., 2000).

The presence of a severe autoimmune syndrome in these mice, despite intact thymic negative selection, points to a defect in peripheral mechanisms of self-tolerance caused by the E2F2 mutation. Even with intact thymic negative selection, a small number of self-reactive thymocytes normally escape negative selection (Van Parijs and Abbas, 1998). Presumably, a failure of peripheral mechanisms of self-tolerance allows these self-reactive lymphocytes to generate autoimmunity in

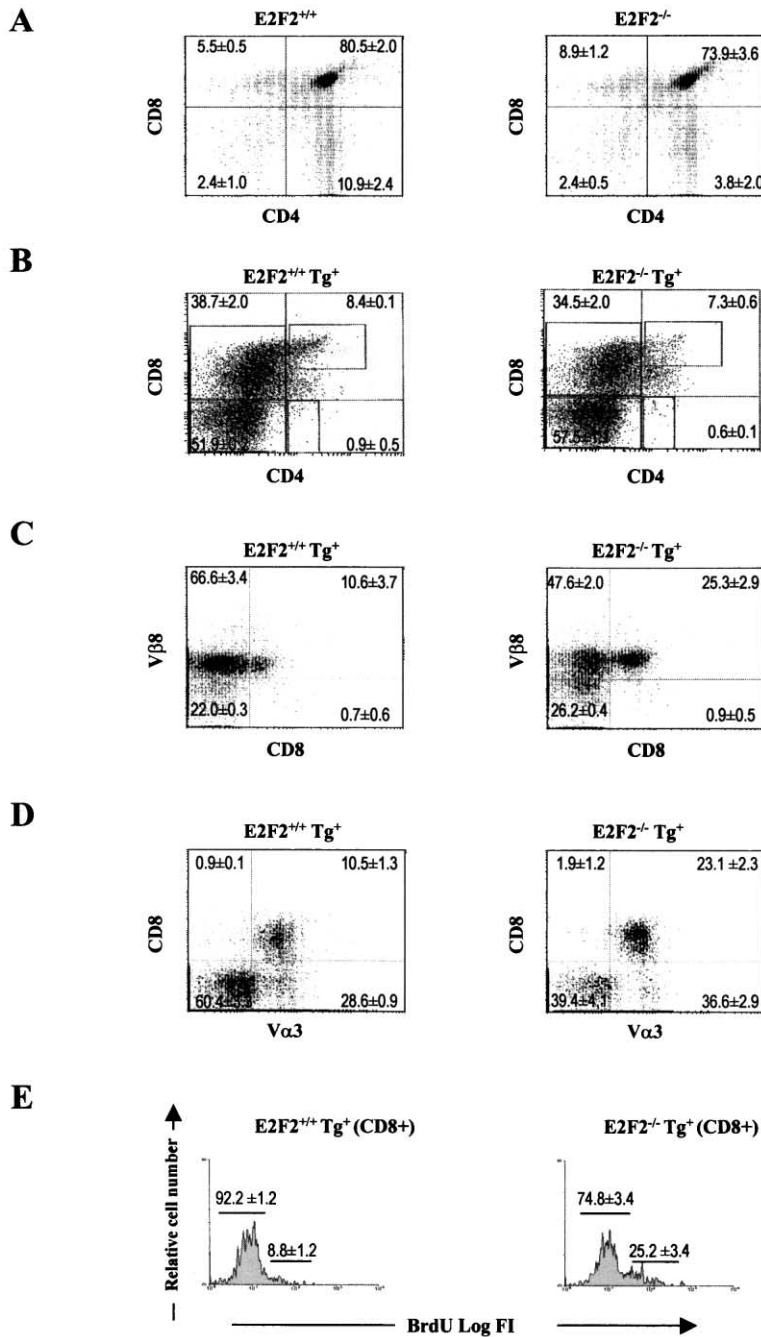


Figure 3. Normal Negative Selection but Increase in the Number of Proliferating E2F2^{-/-} T Lymphocytes Carrying a Self-Reactive TCR-Transgene (Anti-HY)

(A) Thymocyte expression of the CD4 and CD8 cell surface antigens. Thymocytes from wild-type (n = 10) and E2F2^{-/-} mice (n = 10) were double labeled with anti-CD4-FITC and anti-CD8-PE mAbs and analyzed by flow cytometry. The numbers in each quadrant represent the mean percentage of cells in that quadrant.

(B) Thymocytes from E2F2^{+/+} Tg⁺ and E2F2^{-/-} Tg⁺ male mice were labeled as in (A). The experiment shown is representative of two experiments (n = 4 +/+; n = 4 -/-).

(C and D) Lymph node cells from transgenic E2F2^{+/+} and E2F2^{-/-} male mice were double stained with anti-Vβ8.2-FITC and anti-CD8-PE mAbs (C) or with T3.70-biotin antibody, which recognizes the transgenic TCR Vα chain, and anti-CD8-FITC mAbs (D) and analyzed by flow cytometry. The experiment shown is representative of three experiments (n = 6 +/+; n = 6 -/-).

(E) In vivo BrdU labeling of transgenic E2F2^{+/+} (n = 2) and E2F2^{-/-} (n = 2) male mice, followed by an incubation of splenocytes with anti-BrdU-FITC and anti-CD8-PE mAbs, as described in Experimental Procedures.

of the E2F2-deficient mice. The accelerated pace of autoimmunity when the E2F2 deficiency is in the background of the anti-H-Y transgene in male mice presumably reflects the fact that in these mice most T cells that survive to the periphery express an anti-self TCR, specifically the anti-H-Y TCR (Vβ8.2/Vα3).

Expanded Memory T Cell Population Due to E2F2 Deficiency

We reasoned that the accelerated pace of autoimmunity in anti-H-Y E2F2^{-/-} Tg⁺ mice would facilitate the analysis of the tolerance defect in these mice. Examination

of lymph nodes and spleen from anti-H-Y transgenic mice revealed a 2- to 3-fold increase in the proportion of H-Y-specific CD8⁺ T cells in E2F2 mutant animals compared to wild-type controls, as detected by clonotypic mAbs specific for the Vβ8.2 chain and the Vα3 chain of the transgenic receptor (Figures 3C and 3D). Considering the fact that E2F2^{-/-} Tg⁺ male mice also develop marked splenomegaly, the absolute number of potentially self-reactive peripheral T cells was dramatically increased in the E2F2^{-/-} mice.

We measured the proliferation of these Vβ8⁺/CD8⁺ "antigen-primed" cells directly in vivo. To label cells in

S phase, E2F2^{+/+} Tg⁺ and E2F2^{-/-} Tg⁺ mice were given BrdU. These experiments demonstrated a striking increase in the percentage of proliferating CD8⁺ T cells in E2F2^{-/-} Tg⁺ mice compared to E2F2^{+/+} Tg⁺ controls, indicating that a substantial proportion of V β 8⁺/CD8⁺ T lymphocytes were activated in vivo in the E2F2 mutant mice (Figure 3E).

Since the original E2F2-deficient mice (not expressing a TCR transgene) develop similar autoimmune features, albeit more slowly, we examined their peripheral immune system to look for evidence of a similar expansion of the antigen-primed T cell population. We examined E2F2-deficient and wild-type littermates at several ages. The submandibular, axillary, and inguinal lymph nodes of E2F2-deficient mice were of similar size and cellularity as those from wild-type littermates at all ages ($24.1 \times 10^6 \pm 6.8 \times 10^6$ in wild-type versus $22.3 \times 10^6 \pm 4.4 \times 10^6$ in E2F2^{-/-} mice). The spleens of E2F2^{-/-} mice were of comparable size to wild-type at 4 weeks of age but appeared significantly enlarged (more than 2-fold) by 8–12 weeks of age ($1,445 \pm 250$ mg in E2F2^{-/-} mice versus 668 ± 108 mg in E2F2^{+/+} mice) and, as mentioned above, continued to enlarge relative to wild-type through 15 months of age ($3,340 \pm 540$ mg in E2F2^{-/-} mice versus 722 ± 125 mg in E2F2^{+/+} mice).

The ratio of T to B cells was normal in both the lymph nodes and the spleen. However, an increased percentage of CD8⁺ T cells relative to CD4⁺ T cells was found in the lymph nodes and spleen of E2F2-deficient mice compared to wild-type littermates (CD4/CD8 ratio: 1.43 ± 0.3 wild-type versus 0.79 ± 0.1 E2F2^{-/-}) (data not shown). No B220⁺/Thy⁺ cells or CD4⁻/CD8⁻ cells, which are greatly accumulated in autoimmune *lpr* mice, were found in the lymphoid organs of E2F2^{-/-} mice (data not shown).

Next, we analyzed the expression of the lymphocyte activation/memory markers. The fraction of CD8⁺ or CD4⁺ T cells that expressed high levels of CD44 was similar between wild-type and E2F2^{-/-} at 3–4 weeks of age. By 6–8 weeks of age, the CD8⁺ T cell subset in E2F2-deficient mice showed an increased population of CD44^{hi} lymphocytes relative to wild-type littermates. This CD44^{hi} population had expanded further at 15 months of age (Figure 4A). The CD4⁺ T lymphocyte population of E2F2^{-/-} mice also exhibited an increase in the CD44^{hi} population, which was easily detected in lymph nodes and spleens of 15-month-old mice but not in younger mice (Figure 4B). On the other hand, the number of cells expressing high levels of CD69 was only moderately increased in E2F2-deficient mice (Figure 4C). Furthermore, the number of CD69⁺ cells did not increase substantially in older mice. Thus, the majority of the CD44^{hi} lymphocytes that accumulate in the lymphoid organs in E2F2^{-/-} mice are CD69⁻, identifying them as cells with the effector/memory phenotype.

To demonstrate more conclusively that the expanded population of lymphocytes in E2F2^{-/-} mice represents effector/memory cells, we examined their ability to proliferate in response to IL-2 in the absence of any signals delivered through the antigen receptor (London et al., 1999). We found an increase in E2F2^{-/-} lymphocyte proliferation in response to IL-2 relative to wild-type (Figure 4D), which increases with age from twice that of wild-type in 8- to 12-week-old animals to 13 times that of wild-type in 15-month-old mice. The increase in IL-2-

induced proliferation parallels the expansion of the CD44^{hi}/CD69⁻ lymphocyte population, confirming that these are effector/memory cells.

To more directly examine whether T cells from E2F2^{-/-} mice were autoreactive, we performed a syngeneic mixed lymphocyte reaction using irradiated autologous splenocytes as stimulators (Suzuki et al., 2001). Lymph node-derived T cells from E2F2^{-/-} mice responded with enhanced proliferation relative to T cells from wild-type animals when cultured in the presence of syngeneic stimulator cells. Therefore, E2F2^{-/-} T lymphocytes are functionally autoreactive (Figure 4E). Thus, based on the observed characteristic inflammatory infiltrates, the abnormal accumulation of effector/memory cells, the production of anti-dsDNA antibodies, and the presence of anti-self T cells in a syngeneic MLR, we conclude that E2F2-deficient mice develop an autoimmune disease.

Augmented TCR-Stimulated Proliferation of E2F2-Deficient T Cells

To investigate the mechanisms by which E2F2-deficient mice develop autoimmunity, functional studies of lymphocytes from E2F2^{-/-} and wild-type mice were performed. We first determined whether E2F2 plays a role in lymphocyte proliferation. T lymphocytes were stimulated with increasing concentrations of an activating antibody against CD3, and DNA synthesis was measured by [³H]thymidine incorporation 3 days later. As shown in Figure 5A, the incorporation of [³H]thymidine was significantly higher in E2F2^{-/-} T cells compared to wild-type controls ($p < 0.01$). Importantly, this response was more marked at suboptimal concentrations of anti-CD3, suggesting a lower activation threshold in E2F2^{-/-} T cells.

To prove that the enhanced proliferation was intrinsic to naive T cells and was not merely a reflection of the expanded population of effector/memory cells in E2F2^{-/-} mice, we performed an experiment designed to measure the proliferation of purified naive T lymphocytes. For this experiment, we took advantage of the fact that the number of CD44^{hi} cells is similar to wild-type in 4-week-old mice and that the expansion of this population occurs primarily among the CD8⁺ population of T cells. Thus, we purified CD8⁻ T lymphocytes from 4-week-old E2F2^{-/-} mice and littermate controls and examined their ability to proliferate in response to stimulation with anti-CD3. Neither purified E2F2^{-/-} nor wild-type lymphocytes contained detectable CD44^{hi} cells (data not shown). As shown in Figure 5B, naive E2F2^{-/-} T lymphocytes exhibited augmented proliferation relative to wild-type controls, similar to that seen with unpurified T cells. These results indicate that E2F2-deficient naive T cells have an intrinsic proliferative advantage and suggest that E2F2 functions as a negative regulator of T cell proliferation. The enhanced proliferation of naive E2F2 mutant T cells may account, at least in part, for the accumulation of effector/memory cells in E2F2-deficient mice.

To determine whether the enhanced [³H]thymidine incorporation observed in E2F2^{-/-} T cells truly represented an increase in the frequency of cells traversing the cell cycle rather than enhanced survival, we directly measured the fraction of cells in S phase and the fraction undergoing apoptosis. Thus, we stimulated 6- to 8-week-old wild-type or E2F2-deficient lymph node cells with

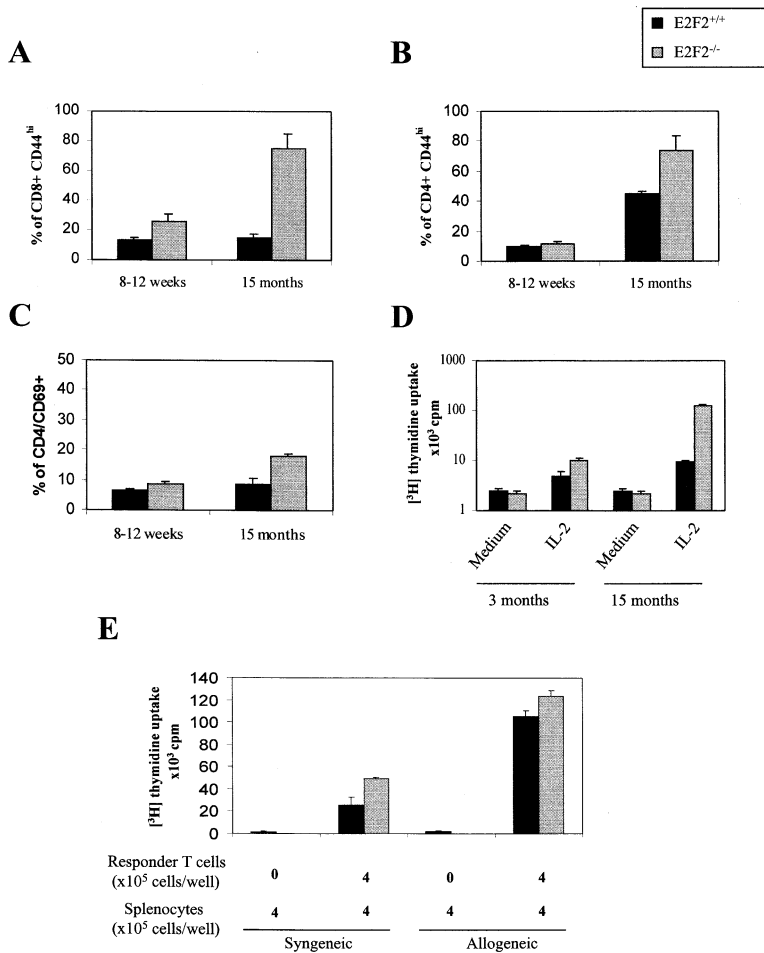


Figure 4. Expanded Memory T Cell Population Due to E2F2 Deficiency

(A and B) Lymph node cells harvested from E2F2^{-/-} and age-matched E2F2^{+/+} mice were double stained with anti-CD44-FITC and anti-CD8-SPRD mAbs (A) or anti-CD44-FITC and anti-CD4-PE mAbs (B) and analyzed by flow cytometry. Histograms show proportions of CD8⁺ or CD4⁺ lymph node cells with CD44^{hi} expression from 8- to 12-week-old mice (n = 5 +/+; n = 5 -/-) or 15-month-old mice (n = 4 +/+; n = 4 -/-). Histogram values in this and subsequent figures indicate the mean ± SEM.

(C) Expression of CD69 activation marker in lymph nodes double labeled with anti-CD69-FITC and anti-CD4-PE mAbs.

(D) Increased response to IL-2 in E2F2^{-/-} mice. Lymph node cells were stimulated with IL-2 as indicated in Experimental Procedures. Proliferation is measured by [³H]thymidine incorporation (n = 4 +/+; n = 4 -/-; two independent experiments).

(E) MLR. Responder T cells from E2F2^{-/-} or E2F2^{+/+} mice were incubated with stimulator cells from syngeneic or allogeneic mice. Proliferation was measured as mean thymidine uptake ± SEM.

anti-CD3 and measured the fraction of cells in S phase (determined by BrdU labeling index) and undergoing apoptosis (determined by TUNEL staining). As shown in Figure 5E, the fraction of cells undergoing apoptosis was similar between wild-type and E2F2-deficient cells. By contrast, as shown in Figure 5D, the fraction of cells staining positively for BrdU was significantly higher in the E2F2-deficient cells. Thus, we conclude that E2F2-deficient cells are hyperproliferative compared to wild-type.

Impaired TCR-Stimulated Proliferation of E2F1-Deficient T Cells

The unexpected result that E2F2^{-/-} T lymphocytes exhibit increased proliferation prompted us to examine the proliferation of E2F1-deficient T lymphocytes. Lymph node T cells from E2F1^{-/-} or wild-type mice were stimulated with antibodies against CD3, and DNA synthesis was measured 3 days after antigenic exposure. In contrast to the results with E2F2-deficient lymphocytes, E2F1-deficient T lymphocytes exhibited decreased anti-CD3-stimulated proliferation compared to wild-type controls (Figure 5C). Thus, E2F1 gene deficiency results in impaired T cell responses, whereas E2F2 gene deficiency results in exaggerated T cell responses. In this case, when we analyzed S phase entry and apoptosis of E2F1^{-/-} T cells, the fraction in S phase was significantly

lower than wild-type, whereas the fraction of apoptotic cells was increased (Figures 5D and 5E). Taken together, these results suggest that E2F1 and E2F2 play different roles in T lymphocyte function.

Normal Response of E2F2-Deficient T Lymphocytes to Apoptotic Stimuli

We next sought to determine whether E2F2 influences peripheral T cell survival in response to other stimuli. E2F2^{+/+} and E2F2^{-/-} T lymphocytes were equally susceptible to cell death following anti-Fas or Dex treatment (data not shown). To test whether accumulation of T cells with a memory phenotype in E2F2^{-/-} mice was due to a failure in activation-induced cell death, we stimulated T cells with Con A for 72 hr, followed by exposure to apoptotic stimuli, including anti-CD3, anti-Fas, serum, and/or IL2 withdrawal. No significant differences between E2F2^{+/+} and E2F2^{-/-} cells were observed in apoptotic responses to any of these treatments (Figure 6). These results indicate that E2F2-deficient T cells show a normal sensitivity to apoptosis.

Differential Gene Expression in E2F1^{-/-} and E2F2^{-/-} T Lymphocytes upon TCR Stimulation

Serum stimulation has been found to induce the expression of E2F1 and E2F2 mRNA in fibroblasts at a time that coincides with the transition of cells from G1 into

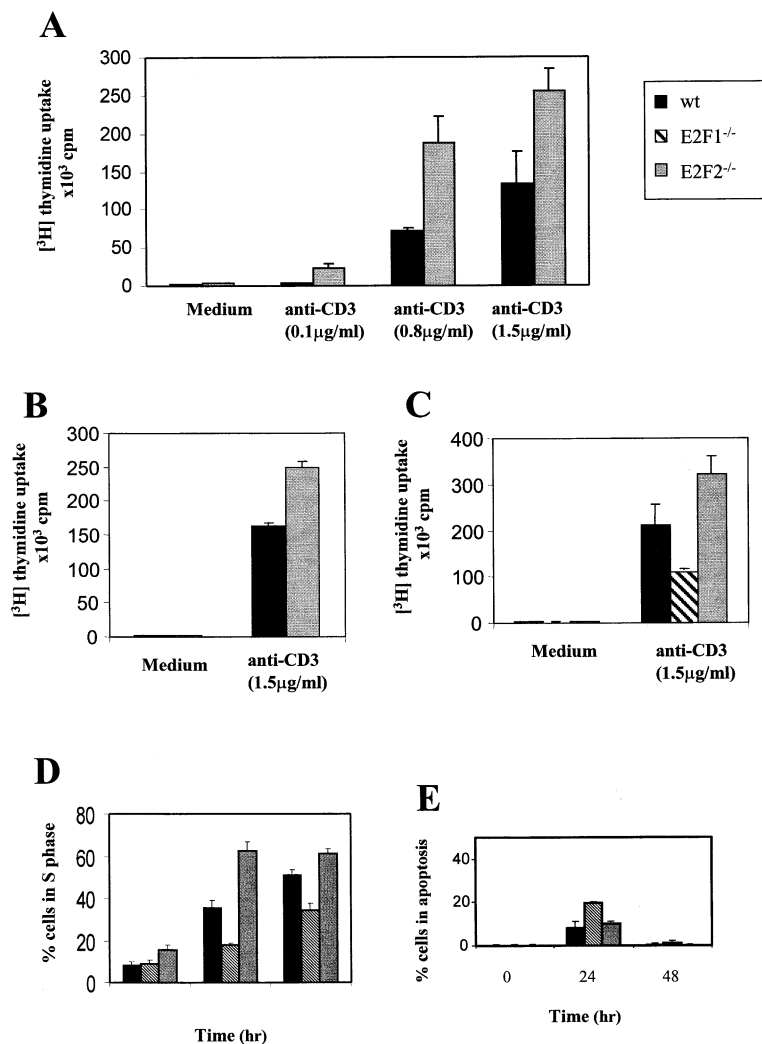


Figure 5. E2F2^{-/-} T Lymphocytes Exhibit a Proliferative Advantage upon TCR Stimulation, Whereas E2F1^{-/-} T Lymphocytes Show an Impaired Response upon Activation

(A) Unpurified lymph node cells from E2F2^{-/-} (n = 6) and E2F2^{+/+} (n = 6) mice were stimulated with anti-CD3, and proliferation rates were determined by [³H]thymidine incorporation. Values represent mean ± SEM of three independent experiments.

(B) Lymph node cells from E2F2^{-/-} and E2F2^{+/+} mice (n = 3 for each genotype) depleted of CD8⁺ cells were stimulated with anti-CD3, as shown in (A).

(C) Lymph node cells from wild-type, E2F1^{-/-}, and E2F2^{-/-} mice (n = 3 for each genotype) were stimulated with anti-CD3, as in (A).

(D and E) Lymph node cells from wild-type, E2F1^{-/-}, or E2F2^{-/-} mice (n = 3 for each genotype) were stimulated with anti-CD3. At various times after stimulation, BrdU incorporation was measured by flow cytometry (D), or apoptosis was measured by the TUNEL assay, followed by flow cytometry (E).

the S (Neuman et al., 1994; DeGregori et al., 1995). The distinct T cell responses that we found in E2F1^{-/-} and E2F2^{-/-} mice upon TCR-mediated activation prompted us to examine the expression profiles of these genes in T lymphocytes obtained from wild-type, E2F1^{-/-}, and

E2F2^{-/-} lymph nodes. As shown in Figure 7A, in wild-type T lymphocytes both E2F1 and E2F2 mRNAs are expressed after TCR stimulation but with differing kinetics; E2F1 mRNA levels peak 24 hr earlier than E2F2 mRNA levels. As with fibroblasts, the timing of expression of E2F1 coincides with the transition into S phase, consistent with a role for E2F1 in the activation of the expression of genes required for DNA replication. By contrast, the delayed expression of E2F2 mRNA is not consistent with a role at the onset of S phase but instead might suggest a role in terminating the proliferative response.

Interestingly, mutation of E2F1 affects the expression of E2F2, and vice versa. The expression of E2F2 mRNA is significantly reduced by mutation of E2F1, suggesting a role for E2F1 as a positive regulator of E2F2 expression, consistent with previous reports (Sears et al., 1997). Unexpectedly, mutation of E2F2 results in enhanced expression of E2F1, suggesting a negative regulation of E2F1 expression by E2F2. Similarly, the expression of *Orc-1* mRNA, known to have E2F sites in its promoter (Ohtani et al., 1996), appeared to be regulated positively by E2F1 and negatively by E2F2 (Figure 7A).

It has been demonstrated previously that E2F sites in

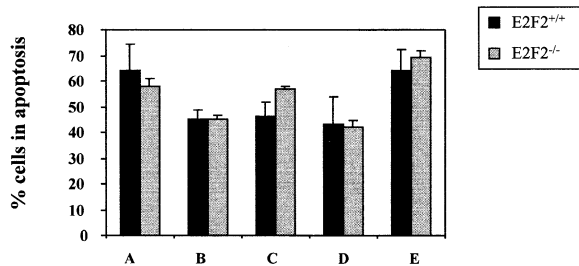


Figure 6. Normal AICD in Peripheral T Cells

Lymph node T cells were activated for 48 hr with Con A prior to treatment with the following apoptotic stimuli: no serum (A), serum (B), anti-CD3 10 μg/ml (C), anti-Fas 1.5 μg/ml (D), or dexamethasone 0.01 M (E). Cell viability was assessed 48 hr later by PI staining followed by flow cytometry.

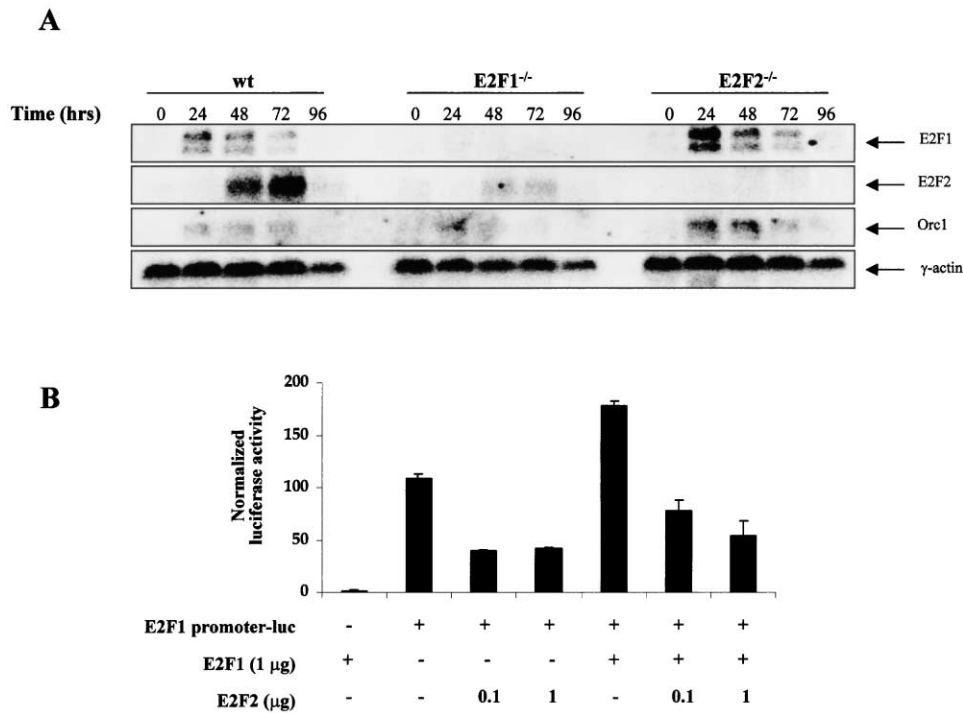


Figure 7. E2F2 Mediates Transcriptional Repression of Genes Required for S Phase

(A) Lymph node cells were harvested from 8- to 12-week-old wild-type ($n = 10$), $E2F1^{-/-}$ ($n = 10$), and $E2F2^{-/-}$ ($n = 10$) mice. Pooled cells were stimulated with Con A ($3 \mu\text{g/ml}$), and RNA was purified at the indicated times. Northern blots were sequentially hybridized with DNA probes to *E2F2*, *E2F1*, *Orc1*, and γ -actin. E2F1 and E2F2 levels for each time point after being normalized to γ -actin mRNA levels were as follows: E2F1 in wild-type cells, 0.4, 1.15, 1.03, 0.76, and 0.5; E2F1 in $E2F2^{-/-}$ cells, 0.56, 1.85, 1.2, 1.1, and 0.83; E2F2 in wild-type cells, 0.35, 0.53, 2.28, 3.4, and 1.7; E2F2 in $E2F1^{-/-}$ cells, 0.54, 0.42, 1.16, 1.65, and 0.75.

(B) E2F2 negatively regulates E2F1 promoter activity. Exponentially growing Jurkat T cells were transfected with various plasmids as indicated in Experimental Procedures. Shown are mean luciferase values \pm SEM, normalized to *Renilla* luciferase activity.

the E2F1 promoter function to negatively regulate the expression of E2F1. The enhanced expression of E2F1 in the context of E2F2 deficiency suggests that E2F2 may at least partially mediate the repression of E2F1 in T lymphocytes. We decided to directly test the ability of E2F2 to repress the E2F1 promoter in T cells. For this purpose, we transfected asynchronously cycling Jurkat T cells with an E2F1 promoter-luciferase reporter construct either alone or with E2F1 or E2F2. As shown in Figure 7B, the promoter-reporter alone had significant basal activity. Cotransfection of an E2F1 expression construct resulted in increased promoter activity, similar to previously published reports in other cell lines (Neuman et al., 1994). In striking contrast, cotransfection of an E2F2 expression construct resulted in a significant and reproducible decrease in both basal promoter activity and E2F1-stimulated E2F1 promoter activity. Thus, both in T cells in vitro and in T cells in vivo, we observe distinctly opposite roles for E2F1 and E2F2.

Discussion

Examination of mice deficient in E2F2, the last of the Rb-specific E2F family members to be purposely mutated in mice, has led to the identification of important nonredundant functions of E2F2. Notably, aged $E2F2^{-/-}$ animals

die early due to autoimmune disease with features of splenomegaly, multiorgan inflammatory infiltrates, glomerulonephritis, and serum anti-DNA antibodies. E2F2-deficient T lymphocytes hyperproliferate in response to TCR stimulation, indicating that E2F2 functions as a negative regulator of cellular proliferation. Furthermore, in striking contrast to E2F1, E2F2 can function in T lymphocytes as a transcriptional repressor of genes containing E2F sites, providing a mechanism for E2F2-mediated suppression of cellular proliferation.

Our investigation into the mechanism of autoimmunity in E2F2-deficient mice has revealed a number of surprising results. First, utilizing crosses with anti-H-Y transgenic mice, we have been able to assess the requirement for E2F2 for normal thymic negative selection, an important mechanism for eliminating autoreactive T cells. We and others have shown previously that E2F1 is required for normal thymic negative selection (Zhu et al., 1999; Garcia et al., 2000). In contrast, in the absence of E2F2, thymocyte maturation and negative selection appear to occur normally. Remarkably, however, the presence of the autoreactive transgenic TCR in these mice results in accelerated autoimmunity. It is worthwhile to note that other gene mutations, including those in CD30 and E2F1 that impair normal thymic negative selection and lead to an expanded population of anti-self T cells, fail to result in autoimmune disease in the anti-H-Y genetic

background (Amakawa et al., 1996; García et al., 2000). These findings, together with our data showing that E2F2 is required for peripheral tolerance, indicate that abrogation of peripheral mechanisms of self-tolerance alone is sufficient to result in autoimmune disease. The importance of peripheral self-tolerance in suppressing autoimmunity has been demonstrated in a number of ways, for example using *lpr* or *gld* mice (Singer and Abbas, 1994; Nagata and Suda, 1995).

The major pathological features of the autoimmune syndrome displayed by E2F2^{-/-} mice are similar to the human disease systemic lupus erythematosus. Interestingly, recent genetic analyses of mice and human lupus patients have identified several lupus susceptibility loci (Shai et al., 1999; Gaffney et al., 1998), including one locus that contains the human E2F2 gene (1p36). Thus, in addition to providing insight into the function of E2F2 and its role in the immune system, these mice might prove to be a useful model for human autoimmune disease.

Our results suggest a straightforward model to explain the breakdown in peripheral tolerance observed in E2F2^{-/-} mice. We find that E2F2-deficient T cells are hyperresponsive to TCR stimulation, responding with increased proliferation to lower concentrations of ligand. Thus, low levels of self-ligands may be sufficient to trigger an autoimmune disease in these mice, resulting in a dramatic, abnormal expansion of the CD44^{hi}/CD69⁻ effector/memory population of T cells. In fact, this expansion of the effector/memory population of T cells is a common feature in mice with autoimmune phenotypes and has also been found in mice with mutations in CTLA-4, IL2R β , Pten, p21, p65^{PI3K}, and PD-1 (Tivol et al., 1995; Waterhouse et al., 1995; Suzuki et al., 1995; Di Cristofano et al., 1999; Nishimura et al., 1999; Balomenos et al., 2000; R-Borlado et al., 2000).

The enhanced TCR-stimulated proliferation of E2F2^{-/-} T cells suggests that, contrary to expectation, E2F2 normally functions to suppress proliferation in naive T cells. To date E2F family members have been implicated as promoters of proliferation. For example, mutation of E2F1 leads to decreased T lymphocyte proliferation (Figure 5), and mutation of E2F3 leads to impaired fibroblast proliferation (Humbert et al., 2000). Interestingly, however, the combined loss of E2F1 and E2F2 leads to increased T cell proliferation, suggesting that simultaneous deficiency of E2F1 and E2F2 activity results in loss of negative function that is dominant to the loss of positive functions (Zhu et al., 2001).

E2F2, in contrast to other E2F family members, may play a crucial role in Rb-mediated repression of genes required for S phase entry, for example, by acting to tether Rb to specific E2F promoter sites. Evidence for this model is provided by the observation that, upon stimulation, E2F2-deficient T cells express increased levels of *E2F1* and *Orc1* mRNA compared to their wild-type counterparts. Furthermore, E2F2 can specifically repress E2F1 promoter activity in T cells in vitro. Thus, in T cells, E2F2 may be the crucial E2F for Rb-mediated gene repression. Possibly the overexpression of *E2F1* or *Orc1* alone is sufficient to account for the enhanced proliferation of E2F2-deficient T cells. However, the enhanced expression of these genes may be merely a sentinel for a myriad of proliferation-promoting genes that rely upon E2F2 for Rb-mediated repression.

The similarities and differences in immune function between the E2F1-deficient and E2F2-deficient mice provide an interesting window on the specialization of members of a gene family. Both E2F1 and E2F2 play important roles in the development of the active T cell repertoire. E2F1 exerts its influence by promoting the apoptosis of immature thymocytes and promoting the proliferation of mature T cells. E2F2, by contrast, exerts its influence by suppressing the proliferation of naive T cells. Together, the two genes appear to act as cassettes whose function can be inserted into an appropriate cell at an appropriate time to promote proliferation, apoptosis, or cell cycle arrest. By functioning in different cells and with different timing, these two genes play quite distinct roles in immune function. E2F1 is required for normal thymic negative selection, the thymic mechanism of self-tolerance. E2F2 is required for normal peripheral mechanisms of self-tolerance. Together, E2F1 and E2F2 appear to function as partners in a dyad of yin and yang. E2F1 promotes T cell proliferation, while E2F2 suppresses T cell proliferation, perhaps in part by suppressing E2F1 gene expression. Furthermore, each functions in its own compartment to maintain self-tolerance. Together the E2F1-E2F2 dyad regulate the major components of immunologic self-tolerance. It remains to be seen to what extent this model of E2F gene function will explain other abnormalities observed in mice deficient in one or more E2F family of genes.

Experimental Procedures

E2F2-Targeting Vector Construction, Embryonic Stem Cell Culture, and Embryo Manipulation

A murine E2F2 genomic clone was isolated from a 129/Sv genomic library using a hybridization probe prepared from an E2F1 cDNA fragment (Kaelin et al., 1992). In addition to the E2F1-containing positives, several plaques were isolated containing crosshybridizing DNA that corresponded to E2F2 (Ivey-Hoyle et al., 1993; Lees et al., 1993). A 3.7 kb fragment containing an exon encoding the DNA binding and activation domain was subcloned into pBlueScript KS(+). To generate a gene-targeting vector, a fragment containing PGK-neo was inserted by blunt-end ligation into the BstXI site within the E2F2 exon. The disrupted E2F2 genomic fragment was then inserted into a backbone containing a PGK-TK gene to provide a negative selectable marker, as previously described (Field et al., 1996).

J1 ES cell culture and injection of ES cells into host blastocysts was performed as previously described (Field et al., 1996).

Southern Hybridization, PCR, and Northern Hybridization

DNA isolated from the offspring of chimeras and subsequent generations was digested with HindIII and used in standard Southern blots, as previously described (Field et al., 1996), using a probe prepared from E2F2 genomic sequences to determine the genotypes of the mice.

A genomic PCR assay to detect the wild-type allele (350 bp) or the mutant E2F2 allele (250 bp) was designed using a common 5' exon primer (TACTGTTCCTGGCCCGC) and a 3' exon primer (TGGAGAGACCCAGGCTG) or a neo^r gene primer (CAAGTGC CAGCGGGCTGCTAAAG). PCR reactions were amplified with Taq polymerase (PE Biosystems) and analyzed on a 1.5% agarose gel. Wild-type and mutant E2F1 alleles were detected as previously described by PCR (García et al., 2000).

For Northern hybridization, cells were isolated as indicated below, and RNA was purified using the TRIzol RNA isolation system (Life Technologies, Inc). The cDNAs used as probes for Northern analyses were: mouse *E2F2* (a 0.6 kb EcoRI fragment), human *E2F1* (a 0.9 kb SalI-EcoRI fragment), mouse *Orc1* (a 2.6 kb HindIII fragment), and γ -actin (a 1.3 kb HindIII-EcoRI fragment). The levels of mRNA expression were visualized and quantitated by a Molecular Imager (BioRad).

In Vitro Lymphocyte Stimulation and Death Assays

Thymuses, lymph nodes, and spleens were harvested and purified from wild-type, E2F1^{-/-}, and E2F2^{-/-} mice as previously described (Field et al., 1996) with minor modifications. To purify a CD8⁻ lymphocyte population, lymph node cells were incubated with biotinylated anti-CD8 antibodies and streptavidin-coated superparamagnetic beads (Dynabeads M-280, Dynal) and negatively selected using a Dynal MPC (Magnetic Particle Concentrator). For analysis of TCR-mediated responses, freshly isolated or purified T lymphocytes from lymph nodes or spleen (1×10^6 /ml) were stimulated with ConA (3 μ g/ml; Sigma) or immobilized antibodies against CD3 (145.2C11, 0.1–1.5 μ g/well; Pharmingen). Forty-eight hours later [³H]thymidine (1 μ Ci/100 μ l) was added, and proliferation was measured 16 hr later. For IL-2 responses, freshly isolated lymphocytes were cultured for 3 days in the presence or absence of conditioned medium (10%) containing supernatant of IL-2-producing X63 cells and pulsed with [³H]thymidine (1 μ Ci/100 μ l) for the last 16 hr of treatment. For MLR, lymph node cells (4×10^5) from 6- to 8-week-old E2F2^{-/-} and E2F2^{+/+} mice were incubated with 4×10^5 mitomycin C-treated (40 μ g/ml for 30 min) spleen cells from syngeneic (C57BL6/J \times 129/Sv) or allogeneic (BALB/C) mice. Cells were pulsed on day 2 with [³H]thymidine and harvested on day 3.

For AICD assays, freshly isolated lymph node cells were stimulated with ConA (3 μ g/ml) for 72 hr. Cells were then washed and further cultured in the presence of stimulators of apoptosis. After 24–48 hr, cells were stained with PI for determination of apoptosis ("DNA-prep" kit, Coulter Corp.). The TUNEL assay was performed according to the manufacturer's instructions (Boehringer Mannheim). Cells were analyzed by flow cytometry.

Transfections and Reporter Assays

Jurkat T cells were cultured in RPMI medium supplemented with 10% FCS. Subconfluent cells were transfected using Lipofectamine Plus (Life Technologies, Inc.) with 5 μ g of pGL2-AN firefly luciferase reporter (Neuman et al., 1994), 2 μ g of pRL-TK, and varying amounts of E2F1 (pRc-CMV-HA-E2F1) and E2F2 (pRc-CMV-HA-E2F2) expression plasmids (Krek et al., 1994). The reporter firefly luciferase activities were measured 48 hr after transfection and were normalized to the transfection efficiency as estimated by the activity of *Renilla* luciferase, constitutively expressed from cotransfected pRL-TK.

Flow Cytometric Analysis and S Phase Labeling

For cell surface staining, all antibodies were conjugated to FITC, phycoerythrin (PE), or biotin, and were purchased from Pharmingen. Biotinylated antibodies were developed with streptavidin-SPRD (Southern Biotechnology). Stained cells were analyzed on a Coulter EPICS XL flow cytometer using system II software (Coulter Corp.).

For in vivo S phase labeling, H-Y Tg⁺/E2F2^{-/-} and Tg⁺/E2F2^{+/+} mice were given water containing BrdU (0.8 mg/ml; Sigma) each day for 8 days. Then, mice were sacrificed, and spleen cells were isolated and fixed in ethanol (95%) and double stained with anti-BrdU-FITC (Becton-Dickinson) and anti-CD8-PE (Pharmingen) mAbs, according to the instructions of the manufacturer. Cells were analyzed by flow cytometry. For in vitro S phase labeling, cells were pulse labeled with 10 μ M BrdU for 20 min, processed, and analyzed as previously described (Field et al., 1996).

Histological and Serological Analysis

Indicated tissues were fixed with 10% formalin in PBS and embedded in paraffin. Sections were stained with hematoxylin and eosin by standard methods. For immunofluorescence studies of deposition of immunoglobulins, 6 μ m frozen kidney sections were processed as described (Balomenos et al., 2000) and stained with an FITC-conjugated goat anti-mouse IgG (Southern Biotechnology). Total antibodies against dsDNA autoantibodies were measured as described (Balomenos et al., 2000) and expressed as relative units against a standard positive serum pool derived from five 4-month-old MRL-*lpr* mice.

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