A Role for E2F1 in the Induction of Apoptosis during Thymic Negative Selection[1]

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Abstract

Thymic negative selection is the process in which maturing thymocytes that express T-cell receptors recognizing self are eliminated by apoptotic cell death. The molecular mechanism by which this occurs is poorly understood. Notably, genes involved in cell death, even thymocyte death, such as *Fas*, *Fas-ligand*, *p53*, caspase-1, caspase-3, and caspase-9, and Bcl-2 have been found to not be required for normal thymic negative selection. We have demonstrated previously that *E2F1*-deficient mice have a defect in thymocyte apoptosis. Here we show that E2F1 is required for normal thymic negative selection. Furthermore, we observed an E2F1-dependent increase of p53 protein levels during the process of thymic clonal deletion, which suggests that E2F1 regulates activation-induced apoptosis of self-reactive thymocytes by a p53-dependent mechanism. In contrast, other apoptotic pathways operating on developing thymocytes, such as glucocorticoid-induced cell death, are not mediated by E2F1. The T lymphocytes that escape thymic negative selection migrate to the peripheral immune system but do not appear to be autoreactive, indicating that there may exist E2F1-independent mechanisms of peripheral tolerance, which protect mice from developing an autoimmune response. We expect that *E2F1*-deficient mice will provide a useful tool for understanding the molecular mechanism of and the immunological importance of thymic negative selection.

Introduction

At its discovery 20 years ago, the process of thymic negative selection was hailed as a major leap in our understanding of the mechanism underlying the immune system's exquisite ability to distinguish self from non-self (1–3). Since that time, relatively little progress has been made in understanding the molecular mechanism underlying this process. Thymic negative selection, the mechanism by which thymocytes bearing self-reactive antigen receptors undergo apoptosis in response to a death signal from neighboring cells, requires TCR[4]-mediated recognition of antigenic peptides associated with major histocompatibility complex class I or II molecules on the surface of antigen-presenting cells and involves thymocytes at the immature CD4/CD8 DP developmental stage or early in the mature single positive stage (1–3). Major advances in our knowledge of thymic selection have resulted from the utilization of transgenic mouse models in which TCRs of known specificity are used. Examples of this approach include the introduction of genes encoding a TCR recognizing the male H-Y antigen in the context of H-2D^b. Thymocytes from these transgenic mice are deleted at or before the CD4/CD8 DP developmental stage when the male H-Y antigen is present (4, 5).

Although the process of negative selection is understood to involve the initiation of apoptotic cell death in thymocytes that express TCRs that recognize self, the details are unclear. Notably, *in vivo* mutational analysis of a number of proteins that originally were expected to be important for thymic cell death, including p53, Fas, Fas-ligand, caspase-1, caspase-3, caspase-9, Bcl-2, and others, has shown that none of these are required for normal thymic negative selection. Indeed, to date only a null mutation of the *CD30* gene has been found to impair negative selection (6). Mutation of CD30, a surface antigen found on Reed-Sternberg cells of Hodgkin's disease and on a variety of non-Hodgkin's lymphoma cells, results in a 5-fold increase in the survival of cells expressing particular TCRs that recognize self-antigens, demonstrating a requirement for CD30 in normal thymic negative selection. The identification of additional examples of genes required for thymic negative selection is crucial for two purposes: (*a*) these genes will help delineate the molecular mechanism by which apoptosis is triggered and executed in thymic negative selection; and (*b*) these mutant mice will provide model systems to investigate the role for thymic negative selection in shaping the immune repertoire.

The E2F transcription factor, although initially described as a factor involved in promoting cell cycle progression, has more recently been found to play an important role in apoptosis (7–10). The *E2F* gene family is comprised of six members that show different affinities for pRB family members.

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[4] The abbreviations used are: TCR, T-cell receptor; DP, double positive; SEB, staphylococcal enterotoxin B; TUNEL, terminal deoxynucleotidyl-transferase-mediated nick end labeling; MAb, monoclonal antibody; PE, phycoerythrin.

E2F1, E2F2, and E2F3 bind with high affinity to pRB, whereas E2F4 and E2F5 bind with high affinity to the pRB-related proteins p107 and p130. A newly identified E2F species, referred to as EMA or E2F6, lacks the Rb-interaction domain and does not physically associate with members of the RB family (8). A variety of genes encoding proteins important for cell proliferation are activated by E2F, and the expression of these genes may be a result of transcriptional activation, as well as derepression (8–10). The target genes include those important for DNA replication (*e.g.*, *Pol* α*, Orcl, Mcm*, and *TS*), control of cell cycle (*cyclin A* and *cyclin E*), proto-oncogenes (*myc* and *myb*), and the RB family (*Rb* and *107*). These studies also show that the individual E2F proteins display distinct specificities in the activation of the target genes (11). Coincident with the differential abilities to activate a large array of endogenous genes that encode proteins important for DNA replication and cell cycle, the E2F family members possess distinct activities and functions in cell growth. For example, ectopic overexpression of various E2Fs (including E2F1, E2F2, and E2F3) in tissue culture cells can drive quiescent cells to enter the S phase of cell cycle, whereas E2F4 and E2F5 show little activity in S-phase induction (11–15). Moreover, E2F1 or E2F4 can function as oncogenes in standard fibroblast cotransformation assays with activated *ras* (16) or participate in the immortalization of primary human keratinocytes (17).

Interestingly, overexpression of E2F1, but not other E2Fs, has the additional property of inducing apoptosis in fibroblast cells grown in low serum (11). Furthermore, it has been shown that inhibition of E2F activity by dominant negative mutants can prevent apoptosis in cultured breast epithelial cells and promote tumor growth in SCID mice (18). More direct evidence of a role for E2F1 in apoptosis is provided by experiments with *E2F1*-deficient mice. Mice mutant for *E2F1* display a defect in apoptosis of CD4/CD8 DP thymocytes, leading to an excess of mature T cells (19). These mice are also predisposed to tumor formation (19, 20), a finding that might reflect a role for E2F1 in limiting hyperplasia and tumorigenesis in specific tissues, and thus, E2F1 would function as a tumor suppressor.

Several reports have shown that E2F1 can induce p53-independent apoptosis (21, 22), although in most cases E2F1 appears to induce apoptosis by a p53-mediated mechanism (23, 24). In addition, overexpression of E2F1, but not E2F2, leads to increased levels of p53, and coexpression of the MDM2 protein blocks both E2F1-mediated apoptosis as well as E2F1-mediated accumulation of p53 (15). The mechanism by which E2F1 regulates p53 is unknown, although it could be mediated by $p19^{ARF}$, a protein that stabilizes p53 and activates p53-dependent transcription (25, 26). $p19^{ARF}$ can be induced by E2F1 (11), and its expression is slightly elevated in $Rb^{-/-}$ cells (27), providing a connection between E2F1 and p53. However, $p19^{ARF}$ induction may not be sufficient for apoptosis, and additional E2F1 targets may be necessary for this process (8).

Recent work indicates that the presence of E2F1 is required for the defects caused by the loss of RB function in many cell types in the developing embryo and suggests that dysregulation of E2F1 function is responsible for these defects (28, 29). The fact that *E2F1* mutation rescued apoptosis occurring in the developing lens and central nervous system, which are dependent on p53, but not in the peripheral nervous system, which is p53 independent, also points toward a requirement for the induction of the p53 pathway for the function of E2F1 in apoptosis (29). Loss of E2F1 function also leads to a complete suppression of apoptosis induced by inactivation of *Rb* in the choroid plexus of mice expressing the Tag_{121} transgene, just as in loss of p53 function. It is important to note that the choroid plexus, lens, and central nervous system are normal in *E2F1*-deficient mice, and it is only after disruption of the pRB/E2F regulatory network by SV40-T or *Rb* loss that this role in apoptosis is unmasked (30).

We have demonstrated previously that mutation of *E2F1* in mice results in a defect in thymocyte apoptosis (19). Here we introduced the *E2F1* mutation into mice expressing a transgene for an H-Y-specific T-cell receptor, a system allowing the assessment of thymic negative selection (6). Our results show that apoptosis during negative selection of immature DP thymocytes is impaired in *E2F1*-deficient mice. We also found that expression of p53 correlates with the extent of negative selection. We conclude that E2F1 plays a crucial role in normal thymic negative selection by a mechanism that appears to involve p53.

Results

Defective TCR-mediated, but not Glucocorticoid-mediated, Apoptosis in $E2F1^{-/-}$ Thymocytes. We have shown previously that *in vitro* cultured $E2F1^{-/-}$ thymocytes demonstrate increased viability relative to their wild-type control cells. Specifically, CD4/CD8 DP thymocytes display an enhanced level of survival in culture (spontaneous cell death), suggesting that E2F1 plays some role in the regulation of apoptosis in this immature population (19). To examine the effect of *E2F1* mutation on apoptosis caused by inducers of cell death that specifically target immature thymocytes, we evaluated the *in vitro* responses to dexamethasone treatment, a glucocorticoid known to cause rapid apoptotic death of cortical DP thymocytes (31, 32). After 18 h of treatment with this agent, there was extensive cell death in culture, but we found no differences in viability between thymocytes from mutant and control mice (Fig. 1). We further investigated thymocyte responses to cross-linking with anti-CD3 antibody, which is known to activate mature T cells but kill immature DP thymocytes *in vitro* and *in vivo* (33). By 24 h after *in vitro* anti-CD3 cross-linking, there were ~30% more live *E2F1*-mutant thymocytes as compared with controls (Fig. 1), consistent with the data obtained by *in vivo* anti-CD3 treatment (19). These results suggest that E2F1 may be selectively involved in the apoptosis triggered through the TCR in immature thymocytes.

Negative Selection in TCR-Transgenic *E2F1*$^{-/-}$ Mice Is Defective. Treatment of thymocytes with anti-CD3 is thought to mimic activation-induced cell death similar to that observed with thymic deletion. Thus, the observation that thymocytes from *E2F1*-deficient mice are not susceptible to anti-CD3-mediated death pointed toward a possible defect in negative selection. Negative selection, the mechanism by

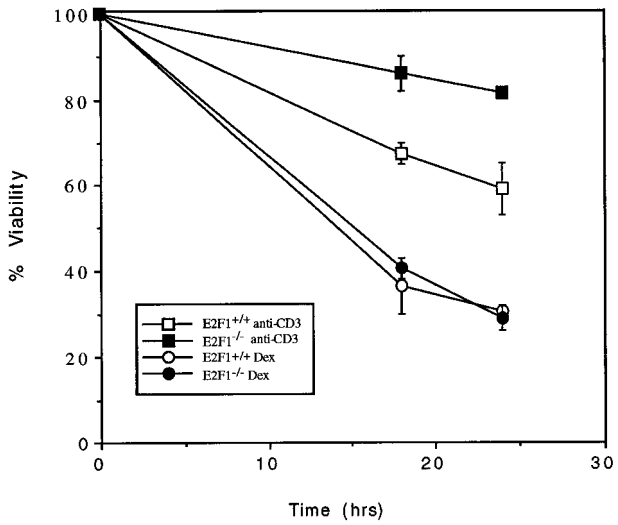

Fig. 1. Defect in apoptosis induced *in vitro* by anti-CD3 of *E2F1*-deficient thymocytes. Thymocytes (1.5×10^6 cells/ml) harvested from adult $E2F1^{+/+}$ ($n = 3$) and $E2F1^{-/-}$ ($n = 3$) mice were cultured in 96-well plates for 18 or 24 h in complete DMEM in the absence or presence of dexamethasone (1 μM; *Dex*) or immobilized anti-CD3 antibody (2 μg/ml). Cell viability was assayed by trypan blue exclusion. No significant differences in viability were observed when comparing untreated $E2F1^{+/+}$ and $E2F1^{-/-}$ thymocytes (data not shown). The values depict the percentage of alive cells in treated cultures compared with that of untreated cultures for each time point. The data shown represent the means for measurements of viability of thymocytes obtained from three independent experiments; *bars*, SE.

which thymocytes that recognize self-antigen are eliminated, results from apoptotic loss of self-antigen-recognizing thymocytes stimulated through the TCR (2, 3).

Ordinarily, negative selection is difficult to observe because only a small fraction of thymocytes undergo negative selection to any particular antigen (34). To increase the fraction of thymocytes undergoing negative selection, we crossed the $E2F1^{-/-}$ mice with a TCR-transgenic mouse line specific for the male (H-Y) antigen, maintaining the H-2^b background. The transgenic receptor is composed of the Vα3 and Vβ8.2 members of the α and β chain variable region gene families, and this TCR is expressed in virtually all T cells in transgenic mice (4). Thymocytes expressing the H-Y-specific transgenic TCRs are positively selected in female H-2^b mice, negatively selected in male H-2^b mice, and nonselected in H-2^d mice (5).

When we analyzed the positive-selecting transgenic female mice, we found that thymocyte phenotypes were similar in $E2F1^{-/-}$ as well as $E2F1^{+/+}$ mice (data not shown). Next, we analyzed negative-selecting transgenic male mice, and similar to what has been reported previously (4), we found that the negative-selecting male mice show a massive reduction in cell number in the thymus because of the deletion of H-Y-specific DP thymocytes by apoptosis. Results obtained from $E2F1^{+/+}$ or $E2F1^{+/-}$ mice were similar for all of the experiments described in this article. In contrast, in negative-selecting male mice carrying an *E2F1* mutation, the reduction in thymocyte number was inhibited, and there was a 50–70% higher cell survival rate in *E2F1*-deficient mice (Fig. 2*a*). The proportion of DP cells was also less affected in the mutant mice, which contained approximately a 3–4-fold increase in DP cells compared with wild-type controls (Fig. 2*b*). Similarly, the percentage of thymocytes that presumably escape from negative selection, expressing the transgenic Vβ8 chain and the accessory molecule CD8, was 3–4-fold higher in *E2F1*-deficient mice (29% *versus* 7%). Thus, these data indicate that H-Y transgenic $E2F1^{-/-}$ mice have a partial, but clearly significant, defect in negative selection in the thymus.

The thymic defect observed in transgenic $E2F1^{-/-}$ male mice suggests that E2F1 regulates negative selection by inducing apoptosis of self-reactive thymocytes. To test this possibility, we performed a fluorescent *in situ* cell death assay (TUNEL) on thymocytes from TCR-transgenic $E2F1^{+/+}$ and $E2F1^{-/-}$ male mice and analyzed apoptosis by flow cytometry, as measured by the fraction of cells labeled by FITC-conjugated dUTP. The percentage of thymocytes undergoing apoptosis is detectable in transgenic $E2F1^{+/+}$ male mice relative to nontransgenic mice. However, this percentage is significantly reduced in H-Y transgenic *E2F1*-deficient thymocytes (Fig. 2*c*), suggesting that E2F1 plays a role in apoptosis during activation-induced cell death of immature thymocytes.

Increased Levels of Undeleted Mature T Lymphocytes in Transgenic $E2F1^{-/-}$ Male Mice. The lymph nodes of H-Y transgenic *E2F1*-mutant mice were also enlarged relative to the *E2F1* wild-type controls. Adult $E2F1^{-/-}$ mice, 6–8 week of age, had ~80% more lymphocytes than age-matched $E2F1^{+/+}$ controls (Fig. 3*a*). We then analyzed the phenotype of these cells by flow cytometry and found that >95% of the T lymphocytes expressed transgenic Vβ8 chain (data not shown). To recognize H-Y antigen, T cells need to express both the TCR transgenes and the TCR-associated molecule CD8. We analyzed expression of transgenic Vβ8 and the accessory molecule CD8 in peripheral T cells from male (H-Y-expressing) wild-type and *E2F1*-deficient mice. We found a 2–3-fold increase in potentially functional H-Y-specific peripheral lymphocytes (*i.e.*, expressing Vβ8 and CD8) in $E2F1^{-/-}$ mice compared with wild-type controls (Fig. 3*b*). These results provide further evidence that self-reactive transgene-expressing thymocytes escape negative selection in the thymus.

To test whether lymph node cells were functionally reactive to mitogenic stimulation, we induced them with the lectin ConA (which activates all T cells) or SEB superantigen (which activates T cells expressing Vβ3, Vβ7, Vβ8, and Vβ17; Ref. 35) and analyzed their proliferative ability, as measured by [^{3}H]thymidine uptake. $E2F1^{+/+}$ and $E2F1^{-/-}$ TCR-transgenic lymphocytes responded to ConA stimulation equally well and to the same level as the nontransgenic lymphocytes (Fig. 3*c*), indicating that the proliferative capacity of these cells remains intact. On the other hand, when we analyzed the T-cell response to SEB, we found differences between the transgenic and nontransgenic lymphocytes. The response to SEB of transgenic $E2F1^{+/+}$ T lymphocytes was greatly inhibited, because virtually all transgenic T cells carry an SEB-reactive TCR, in contrast to only 20–30% in nontransgenic T cells. This is consistent with the idea that transgenic Vβ8-bearing

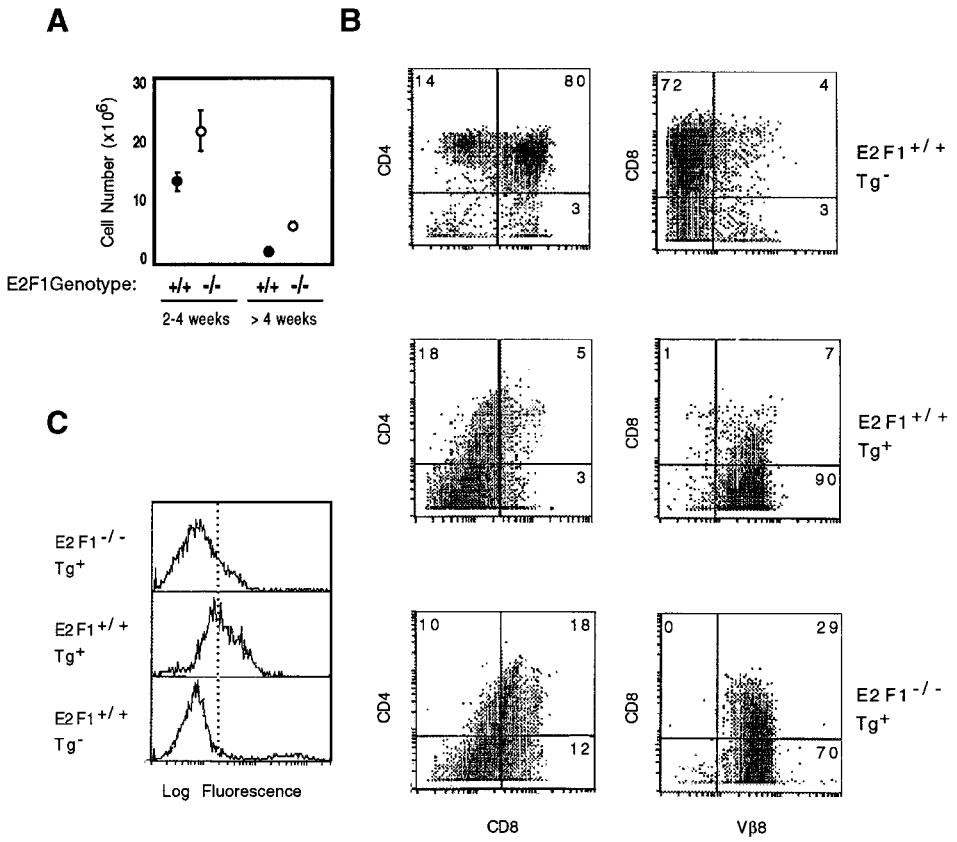

Fig. 2. Clonal deletion of H-Y TCR-transgenic thymocytes is defective in E2F1$^{-/-}$ mice. *A*, thymocytes were harvested from the thymuses of H-Y transgenic *E2F1*$^{+/+}$ ($n = 14$) and *E2F1*$^{-/-}$ ($n = 13$) male mice. The number of thymocytes per thymus was counted and is shown on the graph. *Bars*, SE. *B*, thymocytes from *E2F1*$^{+/+}$ control male mice and *E2F1*$^{+/+}$ and *E2F1*$^{-/-}$ H-Y TCR-transgenic male mice were double stained with anti-CD8-FITC and anti-CD4-PE or anti-Vβ8-FITC and anti-CD8-PE MAbs and analyzed by flow cytometry. In the graphs shown, each dot represents CD4, CD8, or Vβ8 expression levels for a single cell. The cell populations are divided in four quadrants. The numbers in each quadrant represent the percentage of cells in that quadrant. The experiment shown is representative of five experiments comparing in total seven wild-type and seven *E2F1*$^{-/-}$ male mice. *C*, thymocytes from *E2F1*$^{+/+}$ control mice and *E2F1*$^{+/+}$ and *E2F1*$^{-/-}$ H-Y transgenic male mice were harvested and fixed. Apoptosis was assayed using a fluoresceinated *in situ* DNA end-labeling method (TUNEL) as described in "Materials and Methods." Samples were analyzed using a Coulter XL cytometer. Results are representative of the data obtained in two independent experiments.

T cells are anergic to their specific antigen and thus are not autoreactive (4). When we compared the response to SEB in *E2F1*$^{+/+}$ and *E2F1*$^{-/-}$ cells, no differences were found in the response to SEB between T lymphocytes carrying a mutation in the *E2F1* locus and their wild-type controls (Fig. 3*c*), suggesting that TCR-transgenic *E2F1*$^{-/-}$ T cells are not functionally autoreactive, as far as can be determined by this experimental approach.

Induction of Negative Selection by Superantigen SEB Is Impaired in TCR-Transgenic *E2F1*$^{-/-}$ Female Mice. To further examine Vβ8 TCR transgenic *E2F1*-deficient thymocytes for altered sensitivity to negative selection, we analyzed clonal deletion in female transgenic mice by using superantigen SEB. This antigen has been shown to bind to thymocytes expressing Vβ8, leading to a deletion of thymocytes in the DP compartment when high to moderate doses of SEB are injected (35). The intraperitoneal injection of SEB (1.5 μg/gram body weight) led, by 24 h of treatment, to a 75% reduction in the total number of *E2F1*$^{+/-}$ transgenic thymocytes compared with the total number of thymocytes in PBS-injected transgenic females. On the other hand, we only observed a 25% reduction in the number of *E2F1*$^{-/-}$ transgenic thymocytes after SEB injection (Fig. 4). Similar differences were found in the DP compartment. The percentage of CD4/CD8 DP thymocytes was reduced from 60% in PBS-treated animals to 40% in SEB-treated *E2F1*$^{+/-}$ transgenic mice. In contrast, no reduction in the percentage of DP thymocytes was observed in *E2F1*$^{-/-}$ transgenic mice (Fig. 4). These results suggest that E2F1 regulates the negative selection that follows *in vivo* treatment with SEB by inducing clonal deletion of Vβ8-bearing CD4/CD8 transgenic thymocytes.

Reduced Levels of p53 Expression in SEB-treated *E2F1*$^{-/-}$ Transgenic Thymocytes. A number of reports have shown that E2F1 mediates apoptosis by p53-dependent as well as independent mechanisms (29). To begin to understand the mechanism by which E2F1 regulates apoptosis during thymic negative selection, we first analyzed p53 expression in transgenic thymocytes harvested from female wild-type mice that had been subjected to SEB treatment for various time periods. A Western analysis of the samples indicated that there was an increase in the expression of p53 levels in thymocytes 24 h after SEB injection, detectable above the background levels observed in thymocytes injected with PBS alone (Fig. 5*a*). This increase was detectable by 12 h after SEB treatment (data not shown). The higher levels of p53 expression decreased by the second day of the treatment (Fig. 5*a*).

By using this experimental approach, we next examined the levels of p53 expression in *E2F1*-deficient thymocytes after treating mice with SEB for 12 or 24 h. We found that the level of p53 expression was significantly reduced in *E2F1*$^{-/-}$ thymocytes compared with *E2F1*$^{+/-}$ controls at both timepoints analyzed (Fig. 5*b*). The reduction in p53 accumulation correlates with the defect in negative selection observed in SEB-treated E2F1-mutant mice and suggests that E2F1 me-

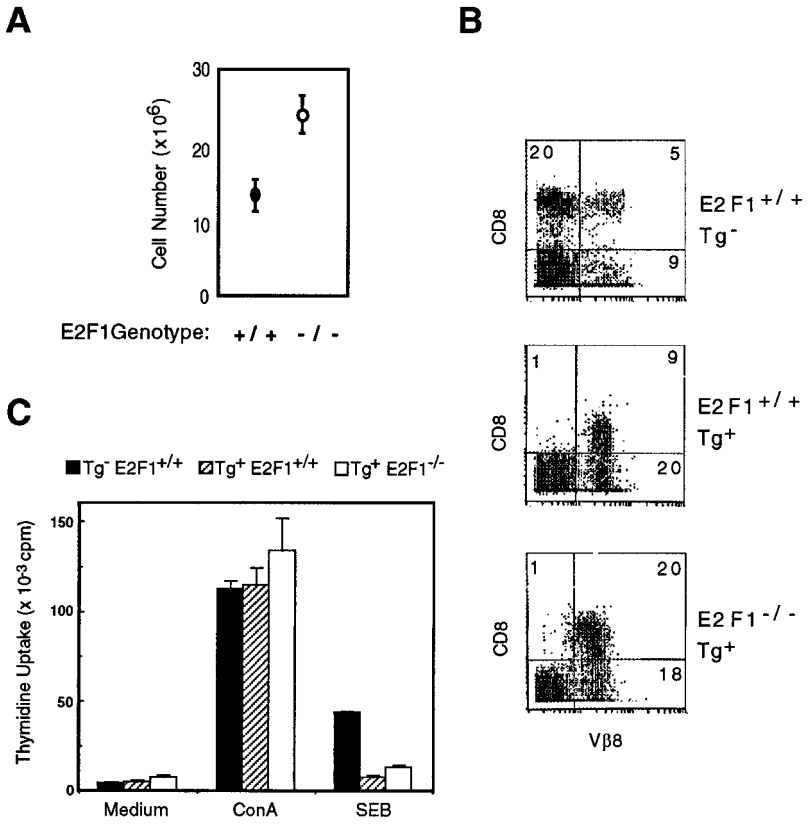

Fig. 3. Enlarged lymph nodes contain higher levels of undeleted T lymphocytes that are not functionally autoreactive. *A*, cells from 12 lymph nodes were harvested from each of six *E2F1*$^{+/+}$ and six *E2F1*$^{-/-}$ 6–8-week-old TCR-transgenic male mice from corresponding anatomical locations and counted, and the average number of cells per mouse was calculated; *bars*, SE. *B*, lymph node cells from *E2F1*$^{+/+}$ control male mice and *E2F1*$^{+/+}$ and *E2F1*$^{-/-}$ H-Y TCR-transgenic male mice were double stained with anti-Vβ8-FITC and anti-CD8-PE MAbs and analyzed by flow cytometry. The numbers in each quadrant represent the percentage of cells in that quadrant. The experiment shown is representative of five experiments comparing in total seven wild-type and seven *E2F1*$^{-/-}$ TCR-transgenic male mice. *C*, lymph node cells from *E2F1*$^{+/+}$ control male mice and *E2F1*$^{+/+}$ and *E2F1*$^{-/-}$ H-Y TCR-transgenic male mice were plated in 96-well flat-bottomed plates at 1.5×10^6 cells/ml and cultured in the absence or presence of ConA (1 μg/ml) or SEB (10 μg/ml). After 3 days, cells were pulsed with 1 μCi of [^{3}H]thymidine/well for 16 h. The data presented are from triplicate cultures ($\pm$SEM) and show one representative example of two independent experiments; *bars*, SE.

diates apoptosis during thymic negative selection by a p53-dependent mechanism.

Discussion

Our results show a requirement for E2F1 during the process of negative selection in the development of T cells. Specifically, E2F1 appears to be required for normal induction of apoptosis in thymocytes bearing TCRs that recognize self-antigens. One of the difficulties of analyzing negative selection comes from the fact that very few apoptotic cells can be detected in the thymus at a given time (34). When TCR-transgenic mice are used, virtually all thymocytes express the transgenic TCR. As a result, if the antigen recognized by this receptor is present, massive cell death occurs, primarily at the CD4/CD8 DP stage of thymic maturation, which makes negative selection more easily detectable. Considering that there are no single experimental approaches to analyze thymic negative selection in all of its complexity (36, 37), we set out to study the role of E2F1 in two negative selection models involving the use of TCR-transgenic animals. In one case, the self-antigen (H-Y) is constantly present in male mice (4, 5). This allows us to analyze not only the role of E2F1 during thymic negative selection but also the effect that the lack of E2F1 may have on the peripheral selection of T lymphocytes. A 3–4-fold higher number of CD8$^+$/Vβ8$^+$ cells can be seen both in the thymus and the lymph nodes of *E2F1*$^{-/-}$ mice compared with wild-type controls, suggesting that the lack of E2F1 protects a proportion of H-Y-specific immature thymocytes from TCR-mediated apoptosis, which would then migrate to the periphery and accumulate there.

The transgenic system used in our study allows us to analyze the role of E2F1 in negative selection using a second type of experimental approach. It consists of analyzing *in vivo* thymic selection induced by superantigen SEB into H-Y TCR-transgenic female mice, where clonal deletion attributable to the presence of the male H-Y antigen is not occurring (5). Similarly to what we found in transgenic males, negative selection in *E2F1*-deficient female mice was also impaired after SEB administration. These results further support the idea that E2F1 regulates thymic negative selection and suggest that E2F1 not only functions to eliminate preneoplastic cells that may have accumulated mutations in genes, such as in *Rb*, but also to eliminate autoimmune T cells.

On the basis of our *in vitro* proliferation data, E2F1-deficient transgenic lymph node T cells from male mice appear to be anergic to the same extent as T cells from E2F1 wild-type controls. This is based on the finding that they respond minimally to SEB (a specific antigen), whereas they proliferate normally after ConA treatment. These results suggest that the transgenic T cells have fallen into an anergic state in the mutant mice, and that some homeostatic process may exist, which is independent of E2F1, to prevent these cells from initiating an autoimmune response. A similar conclusion was reached after the H-Y TCR-transgenic system was used to determine the function of the surface glycoprotein CD30, a member of the tumor necrosis factor receptor

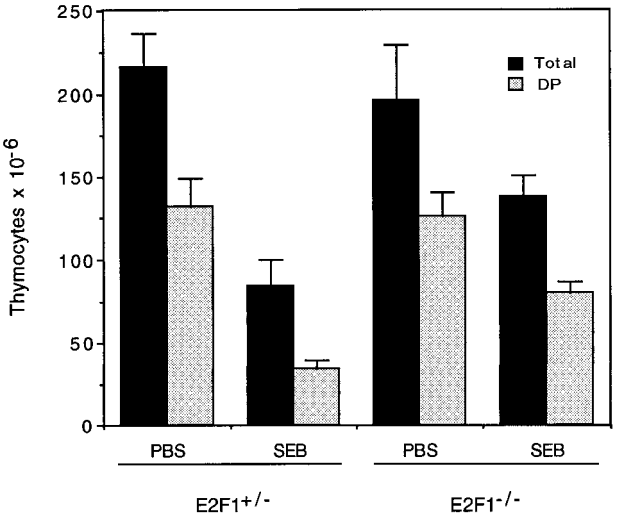

Fig. 4. *E2F1* mutation impairs SEB-mediated deletion in thymocytes. Six-week-old *E2F1*$^{+/-}$ or *E2F1*$^{-/-}$ TCR-transgenic female mice received i.p. injections of PBS or SEB (1.5 μg/gram body weight). After 24 h, thymocytes were harvested and counted. Cells (2×10^5) were stained with anti-CD8-FITC and anti-CD4-PE MAbs and analyzed by flow cytometry. The data shown represent the means of the total number of thymocytes as well as DP thymocytes counted after each treatment and were obtained from two independent experiments; *bars*, SE.

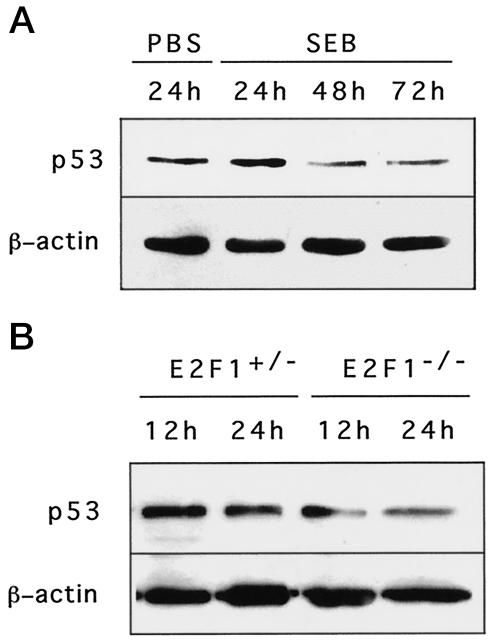

Fig. 5. E2F1 is required for the induction of p53 levels in thymocytes after SEB stimulation. *A*, six-week-old *E2F1*$^{+/+}$ H-Y TCR-transgenic female mice received i.p. injections of PBS or SEB (1.5 μg/gram body weight). Thymuses were harvested at different time points and lysed as described in "Materials and Methods." Protein extracts were separated in SDS-PAGE, blotted to polyvinylidene difluoride membranes, and probed sequentially with anti-p53 and anti-actin antibodies, the last as an internal protein loading control. Protein bands were detected using the ECL system. *B*, six-week-old *E2F1*$^{+/-}$ or *E2F1*$^{-/-}$ TCR-transgenic female mice received i.p. injections of SEB (1.5 μg/gram body weight). After 12 or 24 h, thymocytes were harvested, and cell extracts were prepared and analyzed as in *A*.

family. It was found that CD30 is involved in mediating death signals during negative selection, but that mature cells were not autoreactive (6). Our examination of TCR-transgenic E2F1$^{-/-}$ mice has not revealed any signs of autoimmune disease to date. Nevertheless, we cannot presently rule out the possibility that the CD8$^+$/Vβ8$^+$ cells show some "benign" autoreactivity that could only be revealed *in vivo* and could develop into a more aggressive form in certain conditions where, for instance, lymphokines up-regulate the level of antigen or up-regulate the effector function of these autoreactive T cells (38, 39). Adoptive transfer-type experiments may reveal whether a higher expansion potential of this cell population can be detected in E2F1-mutant compared to wild-type animals.

A variety of conditions induce thymic apoptosis, although the mechanisms by which these conditions induce cell death appear to be different. For example, *bcl-2*-transgenic thymocytes and *caspase-9*-deficient thymocytes display resistance to dexamethasone- and γ-irradiation-mediated death but are sensitive to anti-CD95 antibody (40–42), whereas p35-transgenic thymocytes are resistant to several apoptosis-inducing agents but sensitive to spontaneous cell death *in vitro* (43). It is well established that either TCR engagement or dexamethasone treatment causes specific cell death of immature cortical thymocytes at the DP stage (31, 40), but their apoptosis pathways appear to be different, based on our *in vivo* and *in vitro* results showing that E2F1 is involved in apoptosis mediated through TCR engagement but not in glucocorticoid-induced apoptosis. On the one hand, it is known that dexamethasone-mediated apoptosis is p53 independent (44, 45). On the other hand, our results on SEB-mediated apoptosis correlate p53 levels with the extent of apoptosis, suggesting that p53 mediates self-antigen-induced clonal deletion of immature thymocytes. During thymic development, E2F1 may regulate p53 levels, perhaps by inducing p19^{ARF}, leading to the deletion of self-reactive thymocytes by apoptosis. The extremely low levels of p19^{ARF} that we detect in our system does not presently allow us to determine whether TCR engagement of DP cells leads to an increase in p19^{ARF} levels. However, it is important to note that E2F2 also induces p19^{ARF}, and yet E2F2 does not induce apoptosis (11), suggesting that p19^{ARF} induction is not sufficient for apoptosis, and that additional E2F1 targets are necessary for this process. Although the genes controlled by E2F1 that promote apoptosis are largely unknown, it will be interesting to analyze the expression of p53 regulators (such as p19^{ARF} or Mdm2), as well as the genes regulated by p53 in this system. Additionally, introducing the E2F1 mutation into mice carrying mutations in other regulators of negative selection (such as CD30 or *nur77*) may shed light on the mechanisms that govern this process.

Together with an understanding of the mechanism of E2F1-dependent apoptosis, we believe that *E2F1*-deficient mice provide a useful tool to dissect the molecular mechanism of apoptosis. Furthermore, we expect that these mice will be a useful model for understanding the role of negative selection in shaping the immune repertoire.

Materials and Methods

Mice. *E2F1*$^{-/-}$ (H-2^b) mice were crossed with H-Y TCR (Vα3Vβ8.2) transgenic mice (H-2^b, *E2F1*$^{+/+}$; Ref. 35). F1 mice (H-2^b, *E2F1*$^{+/-}$) were intercrossed to obtain the H-Y TCR/*E2F1*$^{-/-}$ mice. To detect the transgenic H-Y TCR by standard PCR technology, we routinely used the Vβ (AACACATGGAGGCTGCAGTC) and the DJβ (TTCTGCACTGTTATCACCGC) primers. These primers hybridize with the variable region of the β8.2-chain and the VDJ joining region of the β8.2-chain of the transgenic TCR, respectively. The amplified fragment of the transgene is 306 bp long. To detect the wild-type (170 bp) or mutant *E2F1* allele (230 bp) by PCR, we used a common *E2F1* primer (GGATATGATTCTTGGACTTCTTGG) and the *E2F1* wild-type exon primer (CTAAATCTGACCACCAAACGC) or the *neo* gene primer (CAAGTGCCAGCGGGGCTGCTAAAG).

Harvest of Thymocytes and Lymph Node Cells. Thymuses and lymph nodes were mechanically dissociated between two pieces of ground glass. Debris was allowed to settle, and the cells were washed in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 0.5 mM β-mercaptoethanol, 2 mM glutamine, 1 mM HEPES (pH 7.4), and antibiotics (all from Life Technologies, Inc.). Contaminating erythrocytes were removed by hypotonic lysis (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA, pH 7.3) by incubating the cells on ice for 5 min. The cells were washed again in medium and then used in subsequent experiments.

***In Vitro* Apoptosis and T-Cell Stimulation Assays.** Thymocytes were plated in 96-well plates at a concentration of 3×10^5 cells per 200 μl in complete DMEM in the absence (spontaneous cell death) or presence of apoptosis inducers. Some wells were coated overnight with antihamster IgG antibody (1 μg/ml; Pharmingen) at 37°C. Wells were washed with PBS and coated with anti-CD3ε antibody (145-2C11; 2 μg/ml; Pharmingen) for an additional 18-h period at 37°C. Wells were washed again with PBS prior to the addition of the thymocytes. Cell death was assayed at the indicated times after the initiation of culture. Aliquots from replicate wells were mixed with an equal volume of 0.4% trypan blue, and the concentrations of live and dead cells were counted on a hemacytometer. The percentage of cell survival in treated cultures was calculated relative to the percentage survival of parallel untreated cultures.

Lymph node cells (3×10^5) were placed into flat-bottomed 96-well plates and stimulated with 1 μg/ml ConA (Sigma) or 10 μg/ml of SEB (kindly provided by Dr. Ed Palmer, Basel Institute, Basel, Switzerland). Cells were harvested at day 3 after a 16-h pulse with 1 μCi of [^{3}H]thymidine/well. [^{3}H]Thymidine uptake was counted using a gas-phase scintillation counter.

***In Vivo* Apoptosis.** Transgenic female mice (4–6 weeks of age) were challenged with a single i.p. injection of superantigen SEB at a dose of 1.5 μg/gram body weight. Control mice were injected with a similar volume (0.2 ml) of PBS. Thymocytes were isolated at day 1, 2, or 3 and processed for flow cytometric analysis and Western analysis.

Flow Cytometric Analysis and TUNEL Assay. The following MAbs (all obtained from Pharmingen) were used: anti-CD4 (FITC labeled or PE conjugated); anti-CD8 (FITC or PE labeled); and anti-Vβ8 (FITC labeled). Single-cell suspensions of thymocytes and lymph node cells from 3- to 8-week-old male and female mice were washed in PBS and incubated with appropriated MAbs for 30 min on ice. After staining, the cells were washed in PBS, fixed in 2% paraformaldehyde, and analyzed on a Coulter XL cytometer (Coulter, Miami, FL). Dead cells were excluded by gating on forward and side light scatter. For the TUNEL assay, a fluoresceinated *in situ* cell death detection kit was used (Boehringer Mannheim). Briefly, thymocyte suspensions from mice were washed with PBS and fixed in 2% paraformaldehyde. Cells were then permeabilized according to the manufacturer's instructions and incubated with the TUNEL reaction mixture (containing terminal deoxynucleotidyl transferase and FITC-labeled dUTP). Cells were analyzed by flow cytometry.

Western Blot Analysis. Protein extracts were obtained by lysing the thymocytes on lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% NP40, supplemented with protease inhibitors] for 5 min on ice. Lysates were centrifuged at 21,000 $\times$ *g*, and protein concentrations in supernatants were determined using a commercially available kit (Bio-Rad). Thirty μg were loaded per lane, fractionated by SDS-PAGE in 12% polyacrylamide gels, and transferred onto polyvinylidene difluoride membranes (Amersham). Western blots were probed with a MAb against p53 (Ab-1; Oncogene Science) or a MAb against β-actin (AC-15; Sigma). Blots were developed with an horseradish peroxidase-conjugated antimouse IgG antibody (Amersham) secondary antibody and a commercially available chemiluminescence kit, according to the manufacturer's instructions (ECL; Amersham).

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