Thymocyte Maturation Is Regulated by the Activity of the Helix-Loop-Helix Protein, E47

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Summary

The E2A proteins, E12 and E47, are required for progression through multiple developmental pathways, including early B and T lymphopoiesis. Here, we provide in vitro and in vivo evidence demonstrating that E47 activity regulates double-positive thymocyte maturation. In the absence of E47 activity, positive selection of both major histocompatibility complex (MHC) class I- and class II-restricted T cell receptors (TCRs) is perturbed. Additionally, development of CD8 lineage T cells in an MHC class I-restricted TCR transgenic background is sensitive to the dosage of E47. Mice deficient for E47 display an increase in production of mature CD4 and CD8 lineage T cells. Furthermore, ectopic expression of an E2A inhibitor helix-loop-helix protein, Id3, promotes the in vitro differentiation of an immature T cell line. These results demonstrate that E2A functions as a regulator of thymocyte positive selection.

Key words: E2A • positive selection • thymocyte development • helix-loop-helix

During maturation in the thymus, T lineage cells interact with peptide containing MHC proteins either to differentiate or, alternatively, to undergo programmed cell death. Immature double-positive (DP)1 thymocytes that express TCRs possessing high affinity for self-peptide–MHC complexes undergo apoptosis, a process referred to as negative selection. Those cells that express TCRs with sufficient affinity for self-peptide–MHC complexes are induced to differentiate into mature single-positive (SP) T cells through a process termed positive selection. Any DP thymocyte expressing a TCR that is incapable of binding self-peptide–MHC complexes or binding with too low an affinity dies "by neglect" (1–3).

A large number of receptor-mediated signaling pathways and downstream effector molecules that promote positive versus negative selection have been described. However, the nuclear targets of those signaling events remain largely unknown. Previous studies have shown that the helix-loop-helix (HLH) proteins, E12 and E47, are required during the early stages of T lineage development (4). E12 and E47 are encoded by one gene, designated E2A, and arise through alternative splicing of the exon encoding the HLH domain (5, 6). E12 and E47 bind to sequence motifs termed E-boxes, that are found in the regulatory regions of numerous lineage-specific genes (5, 6). In B lymphocytes, E47 homodimeric complexes are the predominant E-box binding species. However, in thymocytes, a portion of the E47 protein binds DNA as a complex with HeLa E-box binding protein (HEB), another HLH protein which is expressed at particularly high levels in developing T cells (7).

Several putative B and T lineage-specific E2A target genes have been described. For example, overexpression of either E12 or E47 in pre-T, macrophage, or pro-B cell lines leads to the induction of the B cell–specific genes L5, V–pre-B, early B cell factor (EBF), and Pax-5 (8–10). Additionally, recombination activating gene (RAG)-1 expression is induced upon ectopic expression of either E12 or E47 in a pre-T or macrophage cell line (9, 10). Another study using enforced expression of Id3, a dominant negative inhibitor of E2A activity, in committed T cell progenitors has also suggested that E2A and/or HEB regulates RAG gene expression (11).

Specific functions of the E2A proteins have been most extensively studied in the context of B lineage development, which is completely blocked at a stage preceding the onset of immunoglobulin gene rearrangement in E2A-deficient mice (4, 12). However, recent experiments have demonstrated that E2A activity is also critical for proper T lineage development. Specifically, E2A-deficient mice are lacking in certain populations of γδ T cells. This deficiency in distinct γδ T cell populations is due, in part, to perturbations in TCR-γδ V(D)J recombination (13). Within the αβ T cell lineage, development is partially blocked at the double-negative stage, before the initiation of TCR β chain

1Abbreviations used in this paper: β2M, β2-microglobulin; BrdU, bromodeoxyuridine; DP, double-positive; EGFP, enhanced green fluorescent protein; HEB, HeLa E-box binding protein; HLH, helix-loop-helix; HSA, heat stable antigen; PDP, peripheral DP; RAG, recombination activating gene; SP, single-positive; WCE, whole cell extract.
gene rearrangement. This early developmental defect likely contributes to the significant decrease in total thymocyte numbers observed in E2A-deficient mice (7). In other studies, overexpression of the negative regulator, Id3, in progenitor thymocytes completely blocked T lineage development (14). We have shown previously that E2A-deficient thymi show increased percentages of mature SP T cells and decreased proportions of immature DP cells (7). These data raised the possibility that the E2A proteins are functionally important beyond the double-negative stage of thymocyte development. Here, we reveal a role for E2A in DP thymocyte maturation. In the absence of E47 activity, an increased proportion of DP thymocytes matures into CD4 and CD8 SP thymocytes. Interestingly, maturation of CD8 SP thymocytes in class I–restricted TCR transgenic mice is sensitive to the dosage of E47, whereas CD4+ thymocyte maturation in class II–restricted TCR transgenic mice is not. In addition, inhibition of E47 DNA binding activity in a DP T cell line by retroviral transduction of a dominant negative inhibitor promotes the maturation of these cells. Taken together, these data suggest that E47 activity contributes to the regulation of thymocyte positive selection.

Materials and Methods

Mouse Strains. The E2A- and E47-deficient mice have been described previously (4, 15). The p2-microglobulin (p2M) mice were purchased from The Jackson Laboratory. All mice were analyzed between 4 and 7 wk of age.

In Vitro Viability Analysis. Total thymocytes from E47- or E2A-deficient mice and wild-type littermates were dissected into RPMI medium containing 10% fetal bovine serum and 50 µM 2-ME and supplemented with glutamine, penicillin, and streptomycin. The cells were stained with antibodies extracellularly as described above and in their drinking water for the indicated times. Thymidine analogue bromodeoxyuridine (BrdU; 0.8 mg/ml) in their drinking water for the indicated times. Thymocytes were labeled with antibodies extracellularly as described above using biotinylated anti-CD4 or anti-TCR-α/β (PharMingen) and PE-labeled anti-CD8 or anti-CD69 (PharMingen). Cells were then fixed and stained with FITC-labeled anti-BrdU (Becton Dickinson) as described previously (16).

Retroviral Supernatant Production and T Lymphoma Transduction. The 16610D9 cell line was derived from a thymoma that developed spontaneously in a p53-deficient mouse and was adapted to culture. The cells were subcloned to generate the 16610D9 line, which was then cultured at 37°C plus 5% CO2 in Optimem (GIBCO BRL) containing 10% fetal bovine serum and 50 µM 2-ME and supplemented with glutamine, penicillin, and streptomycin.

The retroviral vector LZR SpBM N-linker-IR ES-EGFP (S-003) was obtained from Hergen Spits (The Netherlands Cancer Institute, Amsterdam, The Netherlands). The mouse id3 DNA that was cloned into the polylinker was generated by PCR of an id3 cDNA with the following primers: id3 forward, 5′-CCCGAATTCA-TGGACTCACAAGGGACGATGGACAAAGGAATCCATGAA-GGCCTGAGCAGCCGGTG; and id3 reverse, 5′-CCCGAATT-CTCTAGTCGCAAAGCTCCTC, to generate FLAG-tagged id3. The PCR product was cloned into pBSK, digested out of pBSK with EcoRI, and cloned into the EcoRI site in S-003. The adenovirus packaging line (17) was transfected with the retroviral constructs by calcium phosphate precipitation. The transfected cells were switched to 16610D9 culture medium 24 h after transfection, and supernatants were harvested after an additional 24 h. Transduction of the 16610D9 cells was performed as described previously (18). Cells were harvested 48 h after infection and analyzed by flow cytometry as described above using PE-conjugated antibodies (with the exception of CD4, which was Tricolor conjugated), or lysed to make whole cell extracts (WCEs). To prepare WCEs, cells were pelleted, washed once in cold 1% PBS, and the cell pellet was then frozen on dry ice for ~15 min. The pellet was thawed, resuspended in 15 µl/106 cells cold buffer C (20 mM Hepes pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF plus protease inhibitors) containing 1% NP-40, and vortexed vigorously for 2 min at 4°C. Debris was pelleted by spinning at high speed in a microfuge at 4°C, and the supernatant was removed as the WCE. Protein concentrations were determined using Bio-Rad’s protein assay reagent as described by the manufacturer. Double-stranded DNA probes were end-labeled using T4 polynucleotide kinase and purified over a G25 Sepharose column. 15 µg of WCE was used in a gel shift assay as described previously (19). The sequence of the µE5 oligo probe is as follows: 5′-TCGAAGAACCTGCAGCAGCT-3′.

Results

Absence of E2A Leads to Altered Thymocyte Maturation. Previous studies have established that mutant mice lacking both E12 and E47 exhibit abnormalities in thymocyte development (7). To generate sufficient numbers of mice to further characterize the T cell phenotype, we analyzed E47-deficient mice, which have a significantly lower rate of postnatal lethality compared with the E2A−/− mice (15). However, we note that the E47-deficient mice also express reduced levels of the E12 protein compared with control littermates (15). We analyzed thymocytes derived from 4–6-wk-old E2A- or E47-deficient mice for the expression of CD4 and CD8 by flow cytometry. Wld-type and E47 or E2A heterozygous mice had virtually identical thymic profiles (data not shown). However, E2A- and E47-deficient thymi...
showed significant decreases in the percentage of DP thymocytes compared with their heterozygous littermates (Fig. 1 A). In contrast, the proportions of both the CD4 and CD8 SP populations were increased an average of two- to threefold in the E2A- and E47-deficient thymi (Fig. 1, A and B).

Within the thymus, an increase in the relative percentage of mature SP thymocytes can arise through several mechanisms. For example, either aberrant proliferation of the SP populations, increased frequency of apoptosis within the DP population, or increased maturation of the DP cells could explain the phenotypical changes observed in the E2A- and E47-deficient mice. To begin to address these possibilities, we analyzed thymi from E2A-deficient mice for incorporation of BrdU and survival in vitro. E2A-deficient mice and control littermates were injected with BrdU, and the thymic subpopulations were analyzed for BrdU incorporation 24 h after injection. We found no increase in the percentage of SP thymocytes from E2A-deficient mice staining positive for BrdU compared with the control littermates (data not shown). Thus the increased percentage of SP cells is not a result of abnormal proliferation within this population. To determine whether the absence of E2A promotes DP cell death, we isolated total thymocytes from E47- or E2A-deficient mice and wild-type littermates and analyzed the viability of the cells after 28 h in culture. Whereas an average of 52% of the wild-type DP cells was alive after the culture period, only ~17% of the E47- and E2A-deficient DP cells survived (Table I, and data not shown). Similar percentages of wild-type or E2A-deficient CD4+ cells were alive after 28 h in culture, suggesting that the increase in cell death is specific to the DP population (Table I). Thus DP thymocytes lacking E2A activity display decreased survival in vitro.

To address the possibility of an increase in DP thymocyte maturation, we further characterized the changes in the T cell populations by analyzing thymocytes from E2A- and E47-deficient mice for the expression of TCR-α/β and CD69. During normal thymocyte development, expression of both TCR-α/β and CD69 increases upon positive selection (20–24). However, whereas SP thymocytes retain high levels of TCR-α/β expression, CD69 levels are subsequently downregulated upon completion of maturation (21, 24, 25). Thus, TCRhiCD69− cells represent those thymocytes in the process of positive selection, whereas TCRhiCD69+ thymocytes have completed the maturation process. We find that thymocytes derived from E2A- or E47-deficient mice have a three- to fourfold increase in the proportion of TCRhiCD69+ cells compared with their littermates (Fig. 2, and data not shown). The absolute number of mature TCRhiCD69+ thymocytes is increased an average of 1.5–3-fold (data not shown). These data indicate that the absence of E47 leads to an increase in the proportion of SP cells that have completed thymocyte maturation, and suggest that E47 might have an additional role in regulating DP thymocyte differentiation.

### Table I. In Vitro Viability of DP and CD4+ Thymocytes from E2A-deficient Mice and Wild-type Littermates

| Exp. | Percent DP | Percent CD4+ |
|------|------------|--------------|
|      | +/+        | −/−          | +/+  | −/−  |
| 1    | 44         | 15           | 74   | 79   |
| 2    | 56         | 14           | 85   | 82   |
| 3    | 55         | 23           | 92   | 100  |
| Ave. | 52 (± 6)   | 17 (± 5)     | 84 (± 9) | 87 (± 11) |

Total thymocytes from E2A-deficient mice or wild-type littermates were stained with antibodies against the CD4 and CD8 coreceptors and with propidium iodide before culture and after 28 h in culture. The percentage of viable DP or CD4+ cells for three independent experiments is shown. The average (Ave.) for the three experiments (Exp.) is indicated, with the SD in parentheses.

Figure 1. Increased percentage of SP thymocytes in E2A- and E47-deficient mice. (A) Two-color flow cytometric analysis of thymocytes from 4–6-wk-old E2A- and E47-deficient mice and heterozygous littermates. Thymocytes were analyzed by staining with anti-CD8a and anti-CD4. The numbers in each quadrant indicate the percentage of thymocytes in that population. (B) The percentage of mature CD4+ (left) or CD8+ (right) thymocytes is plotted for eight to nine individual E2A- and E47-deficient mice and control littermates. The horizontal bars indicate the average percentage of CD4+ or CD8+ thymocytes for each genotype.
whether the absence of E47 leads to an increase in thymocyte maturation, E47-deficient mice were crossed to AND TCR mice which express a class II–restricted TCR transgene. Maturation of CD4 SP thymocytes is strongly enhanced in H-2b mice expressing the AND TCR (26). Thymocytes derived from E47-deficient mice and wild-type or heterozygous littermates carrying the AND transgene were analyzed by flow cytometry for the expression of CD4 and CD8. As expected, E47+/−;AND mice contained a high fraction of mature CD4+ cells and a correspondingly lower fraction of immature DP thymocytes than nontransgenic thymi (Fig. 3, top panels). Thymic profiles from E47+/−;AND mice were not significantly different from the E47+/+;AND profiles (Fig. 3, top panels). However, the percentage of mature CD4 SP cells was significantly higher (87 vs. 73%) in thymocytes derived from E47−/−;AND mice, and the proportion of DP cells was significantly lower (10 vs. 22%; Fig. 3, top panels). Importantly, we observed a similar trend in the peripheral T cell populations. The ratio of CD4+ to CD8+ splenic T cells increased from ~22 in the wild-type or heterozygous mice expressing the AND TCR to 55 in the E47−/−;AND mice (Fig. 3, middle panels). In the lymph nodes, the ratio of CD4+ to CD8− cells increased from 11 in the E47+/−;AND mice to 69 in the E47−/−;AND mice (Fig. 3, bottom panels). The increase in total percentage of T cells in the E47-deficient lymph nodes (98 vs. 55%) is due to the complete absence of B cells in the E2A- and E47-deficient mice (4, 15). Thus, there is a selective increase in only those SP cells expressing the relevant TCR, and this increase is reflected in the periphery. These data suggest that the absence of E47 can markedly enhance the positive selection of class II–restricted TCRs.

To determine whether the absence of E47 also promotes increased selection of class I–restricted TCRs, we analyzed E47-deficient mice expressing the H-Y TCR transgene, which specifies reactivity with the male H-Y antigen presented by the H-2D b class I molecule (27, 28). Expression of the H-Y TCR transgene in females of the H-2b background leads to positive selection of CD8 SP thymocytes (27, 28). In contrast, thymocytes in male mice expressing the H-Y TCR are deleted at the DP stage. Negative selection mediated by the H-Y TCR transgene was unaffected by the absence of E47 (data not shown). However, E47−/−;H-Y female mice displayed an increased percentage of mature CD8 SP thymocytes compared with the E47 heterozygous littermates (56.6 vs. 33.6%; Fig. 4 A). This phenotype was accompanied by a significant decrease in the proportion of immature DP thymocytes (29.6 vs. 46%; Fig. 4 A). Be-
cause of continued rearrangement of endogenous α chain genes in TCR transgenic mice, a fraction of the thymocytes will express a TCR composed of the transgenic β chain and an endogenous α chain (25, 27, 29). To analyze the proportion of cells expressing the transgenic TCR-α/β, we stained total thymocytes with the T3.70 antibody, which reacts specifically with the transgenic α chain molecule (30). Thymocytes isolated from E47-/-;H-Y mice contained...
The increased percentage of mature CD8+ T cells observed in the E47−/−; H-Y mice is also reflected in the periphery. The ratio of CD8+ to CD4+ T cells is increased from ~0.4 in the E47+/−; H-Y spleen to 1.2 in the E47−/−; H-Y spleen (Fig. 4 B, top panels). A similar change in the CD8/CD4 ratio was observed in the lymph nodes (Fig. 4 B). Therefore, like the E47−/−; AND mice, the E47−/−; H-Y mice display an increase in only the percentage of cells expressing the relevant TCR, and the relative proportions of CD4 and CD8 lineage cells are altered in the peripheral lymphoid organs as well. These changes in peripheral CD8/CD4 ratios from E47-deficient/TCR transgenic mice would not be expected if the increase in proportion of thymic SP cells was entirely the result of increased death within the DP thymocyte population. In addition, a selective increase in only the population of cells expressing the relevant TCR is not consistent with a defect in emigration of mature SP thymocytes from the thymus. Interestingly, in addition to the increase in CD8 SP cells, E47−/−; H-Y transgenic mice contained elevated percentages of DP cells that expressed CD69 and high levels of TCR-α/β (Fig. 4 C). Thus, a higher proportion of the DP population is undergoing positive selection in the absence of E47. Taken together, these data suggest that the absence of E47 results in increased selection to both class I- and class II-restricted T cell receptors.

As described above, heterozygosity at the E47 locus does not perturb maturation of SP thymocytes expressing class II-restricted TCR transgenes. In contrast, we consistently observed increases in the proportion of CD8+ cells in female H-Y transgenic mice that were heterozygous for E47 compared with wild-type littermates (Fig. 4 D). In most cases, the percentage of CD8+ cells was increased in the periphery of E47−/−; H-Y mice as well (Fig. 4 D). Thus, maturation of CD8 SP cells in mice expressing the H-Y TCR transgene is influenced by the dosage of E47.

A absence of E2A leads to the appearance of DP cells in peripheral lymphoid organs of H-Y transgenic mice. In addition to the increased production of CD8 SP cells, female E47−/−; H-Y mice displayed an aberrant population of T cells in the peripheral lymphoid organs that expressed high levels of CD8 and intermediate to high levels of CD4 (Fig. 4 D). To examine whether the peripheral DP (PDP) population expressed markers characteristic of mature T lineage cells, we analyzed this population for the expression of CD48, CD8, and HSA (Fig. 5 A). The CD4 versus CD8 profiles for the thymocytes (top panels) and splenocytes (bottom panels) are shown (A), and the numbers in each quadrant indicate the percentage of cells in that population. The boxed regions in the CD4 versus CD8 plots (a–e) indicate the analysis gates used to define the populations analyzed in B and C. In the histogram plots (B and C), the lettered arrows indicate the staining pattern for the corresponding boxed populations in A. To make comparisons clearer, the histograms were plotted in pairs to compare the peripheral DP population with the thymic DP population (left panels) and with the SP populations (right panels).

Figure 5. Appearance of DP cells in the periphery of E47-deficient/H-Y transgenic mice. 6-wk-old H-Y transgenic mice of the indicated E47 genotype were analyzed by three-color flow cytometry for the expression of CD4, CD8, and either HSA (B) or TCR-α/β (C). The CD4 versus CD8 plots for the thymocytes (top panels) and splenocytes (bottom panels) are shown (A), and the numbers in each quadrant indicate the percentage of cells in that population. The boxed regions in the CD4 versus CD8 plots (a–e) indicate the analysis gates used to define the populations analyzed in B and C. In the histogram plots (B and C), the lettered arrows indicate the staining pattern for the corresponding boxed populations in A. To make comparisons clearer, the histograms were plotted in pairs to compare the peripheral DP population with the thymic DP population (left panels) and with the SP populations (right panels).
ratio from 146 in the E47−/−;β2M−/− spleen to 37 in the E47−/−;β2M−/− spleen indicates that the absence of E47 allows for an increase in maturation of CD8+ cells even in the absence of the appropriate TCR–MHC interaction. Thus, the data suggest that the downregulation of E2A activity can promote thymocyte maturation.

Absence of E47 Leads to an Increased Production Rate of Mature SP Thymocytes. The steady state size of the thymic subpopulations is determined by the rate at which cells are generated, the rate at which they die, and the rate at which they emigrate from the thymus. To more directly assess the effect of an E47 deficiency on the production of mature SP T cells, we measured the kinetics of appearance of mature thymocytes using the thymidine analogue BrdU. We exposed E47-deficient mice and their heterozygous littermates to BrdU in their drinking water and then analyzed for the presence of BrdU and the expression of TCR-α/β and CD69 (Fig. 7, A and B). Because cells expressing medium to high levels of TCR are not dividing, there is a lag in the appearance of BrdU in these populations. As described above, both the E47-deficient and control mice display this lag in labeling of mature thymocytes using the thymidine analogue BrdU. We exposed E47-deficient mice and their heterozygous littermates to BrdU in their drinking water and then analyzed for the presence of BrdU and the expression of TCR-α/β and CD69 (Fig. 7, A and B). Because cells expressing medium to high levels of TCR are not dividing, there is a lag in the appearance of BrdU in these populations. As described above, both the E47-deficient and control mice display this lag in labeling of mature thymocytes, demonstrating that the absence of E47 does not lead to the aberrant proliferation of the SP cells. Because the TCRmed-hi populations are nondividing, the rate of appearance of BrdU in these populations reflects the rate at which these cells are produced. In mice deficient for E47, the rate of appearance of BrdU-labeled TCRhiCD69+ mature thymocytes is significantly enhanced, suggesting that the relative proportion of cells that develop into mature CD69+ T cells is increased in the absence of E47 (Fig. 7 B). In contrast, E47-deficient mice display a decrease in the rate of appearance of BrdU in the TCRmed CD69+ population (Fig. 7 B). Taken together, these data suggest that the rate at which DP thymocytes mature into TCRhiCD69+ SP T cells is enhanced in the absence of E47.

Inhibition of E47 Binding Activity Promotes T Cell Differentiation. As shown in Table I, E2A-deficient DP thymocytes show reduced survival in vitro. However, the data described above suggest that the absence of E47 also promotes thymocyte positive selection. To test directly whether lowering the activity of E47 promotes maturation, we used a retroviral transduction system to study the effects of inhibiting E2A activity in an immature DP T cell line. The mouse cDNA for Id3, a negative regulator of E2A binding activity, was cloned into the retroviral vector S-003 (14). This vector allows translation of both Id3 and enhanced green fluorescent protein (EGFP) from one retroviral transcript. Thus, retroviral transductants can be identified by the expression of EGFP. The S-003/Id3 and S-003 empty vector constructs were transfected into the φX-eco retroviral packaging line, and supernatants from these cells were used to transduce virus into the T cells. The 16610D9 T

Figure 6. Development of CD8+ cells in the E47-deficient mice requires MHC class I expression. E47-deficient mice were bred onto the β2M−/− background, and 4–6-wk-old littermates were analyzed for CD4, CD8, and TCR expression. Thymocytes (A) and splenocytes (B) from E47-deficient and heterozygous littermates on a β2M−/− or heterozygous background were analyzed by staining with anti-CD8α and anti-CD4. The numbers in each quadrant indicate the percentage of thymocytes in that population. The CD4/CD8 ratios for the spleens are indicated under the FACS® plots in B.
cell line used was derived from a thymoma that developed spontaneously in a p53-deficient mouse and was adapted to culture. This T cell line expresses characteristics typical of DP thymocytes, including high levels of HSA, intermediate levels of TCR, and low levels of CD5 and CD44 (Fig. 8 C). In addition, the 16610D9 cells express significant levels of E2A binding activity (Fig. 8 A).

Id3 was transduced into the 16610D9 line and analyzed 48 h after infection for the expression of HSA, CD69, CD44, CD5, TCR- and CD8. The early stages of positive selection are marked by increased expression of both TCR- and CD69 surface expression. (A) Representative TCR- and CD69 FACS® plots for E47-deficient and heterozygous mice. (B) Rate of appearance of BrdU-labeled TCR+ and CD69+ cells. (C) The percent BrdU-labeled cells of total cells is plotted against the time of continuous exposure to BrdU. The gates used to define the TCR+CD69+ cells and TCR+CD69+ cells are shown as R1 and R2, respectively. The numbers plotted are the averages from two mice.

Discussion

Immature thymocytes have the ability to undergo two distinct fates. A DP thymocyte can be selected to mature into either a CD+ or CD8+ T cell, or alternatively it may undergo cell death. Although a large number of signaling molecules have been implicated in thymic selection, their nuclear targets have remained largely unknown. Here, we show that the activity of one particular transcriptional regulator, E47, is important in regulating thymocyte positive selection. Our data indicate that the relative level of E47 can influence thymocyte selection to both the CD4+ and CD8+ lineages. In the absence of E47 activity, the rate of production of TCR-CD69+ thymocytes is enhanced, resulting in increased percentages of SP thymocytes. In a TCR transgenic background, a deficiency in E47 results in an increase in positive selection of only those T cells expressing the relevant TCR. In addition, inhibition of E47 activity within a DP T cell line induces the cells to differentiate. Based on these observations, we propose that the downregulation of E2A activity promotes thymocyte maturation.

A Role for E47 in Controlling Thymocyte Maturation

Mice deficient for E2A display an increased percentage of mature SP thymocytes coupled with a decreased proportion of DP cells. Here we show that the E2A-deficient mice exhibit increased DP cell death in vitro. Although it is likely that a decreased rate of DP survival could contribute to the observed phenotype, it is doubtful that the increase in proportion of SP cells is solely a result of increased death within the DP population. Our kinetic data demonstrate that the absence of E2A activity additionally promotes thymocyte maturation. Furthermore, E47-deficient TCR transgenic mice display a corresponding increase of SP T cells expressing only the relevant TCR in both the thymus and
The appearance of DP T cells expressing characteristics of positively selected cells in the periphery of E47−/−;H-Y mice also indicates that the absence of E2A affects DP thymocyte maturation. Moreover, our data show that CD8+ T cells have the ability to mature in the absence of class I, albeit with low efficiency, when E47 is lacking. Finally, inhibition of E47 activity within a DP T cell line alters the expression of markers that correlate with DP maturation. Taken together, these data demonstrate that a downregulation of E47 activity promotes thymocyte maturation and that a combination of increased DP cell death and enhanced maturation contributes significantly to the phenotype observed in the E47-deficient mice.

This raises the question of how E47 is involved in positive selection. TCR–MHC interactions that induce positive selection must have two important effects. These signals must rescue the cells from apoptosis and promote maturation. Recent data indicate that the restoration of E47 activity in E2A-deficient DP thymoma cell lines leads to programmed cell death (18). It is possible that the continued presence of E2A activity in DP thymocytes that have received a signal through the TCR contributes to the induction of apoptosis. Thus, only those cells that downregulate E2A activity survive. We note that the thymic phenotype of E47-deficient mice shows similarities to the bcl-2 transgenic mice, including a higher percentage of CD8 SP cells and an alteration in the CD4/CD8 ratio (7, 35–38). Like bcl-2 expression, decreased E47 activity might prolong the survival of the DP thymocytes, thus leading to enhanced production of CD8+ cells. However, as mentioned above, DP thymocytes lacking E2A show an increase in cell death in vitro, whereas bcl-2 expression prolongs DP thymocyte survival (36, 38). Additionally, expression of bcl-2 promotes CD8+ thymocyte maturation in MHC class I-deficient mice and in class II-restricted TCR transgenic mice, whereas the absence of E47 does not (35; Figs. 3 and 6). Finally, in contrast to the E47-deficient mice, the excess CD8+ thymocytes that develop in the bcl-2 transgenic mice do not survive in the periphery (35, 36).

Recently, activation of the Notch signaling pathway has been shown to upregulate several markers that correlate with
DP maturation. In particular, a constitutively active fragment of Notch was capable of upregulating bcl-2 expression in DP thymomas and rendering these cells resistant to glucocorticoid-induced apoptosis (39). Interestingly, like the E47-deficient mice, activated Notch1 transgenics have increased percentages of CD8+ thymocytes (40). However, a significant population of CD8+ thymocytes can develop in Notch1 transgenic mice lacking expression of class I MHC, whereas only a small CD8+ population can develop in class I-deficient mice lacking E47 (40). N onetheless, it is possible that Notch signaling regulates E2A activity. In fact, others have shown that activated Notch1 and Notch2 effectively inhibit E47 activity in transient transfection assays (41).

It is conceivable that the lack of E47 activity lowers the threshold of avidity required for positive selection. This model has been proposed to explain the phenotype of the activated Notch1 transgenic mice (39). For example, the absence of E47 might allow for the development of T cells that would normally die by neglect because of an insufficient affinity for MHC. It is thought that CD8 lineage commitment requires weaker signals from the TCR compared with the strength of the signal required for CD4 lineage commitment (42, 43). If less stringent signals are required for commitment to the CD8 lineage, then a slight lowering of the threshold affinity of positive selection may result in an even greater increase in CD8+ cells compared with CD4+ cells. Such a model might explain the decrease in the CD4+/CD8+ thymocyte ratio in E2A-deficient mice and the finding that more CD8+ T cells develop in MHC class I-deficient mice that also lack E47 expression. Thus, the absence of E47 might allow for the development of some T cells that would normally die by neglect.

E47 Target Genes. The data described above suggest that E47 activity is downregulated during the final stages of thymocyte maturation. A key step during the transition of a DP to SP thymocyte is the termination of TCR-αβ rearrangements. Previous studies have suggested a link between E2A activity and RAG expression, which is also downregulated in immature thymocytes upon interaction of the TCR with the appropriate MHC–peptide complex (44). For example, expression of both RAG-1 and RAG-2 can be activated in a pre-T cell line by the ectopic expression of E47 (9). Additionally, E2A and HEB have been implicated in regulating RAG transcription in committed T lineage cells (11). Consistent with this idea, E2A-deficient thymocytes show decreased levels of both RAG-1 and RAG-2 transcripts (Bain, G., unpublished observations). However, RAG levels are not completely abolished, and TCR V(D)J recombination proceeds in E2A-deficient mice, presumably due to the presence of an HEB homodimeric complex detectable in E2A-deficient thymocyte extracts (7). We would like to suggest that an interaction of TCR with the appropriate peptide–MHC complex results in the downregulation of total HLH activity, which leads to the abatement of RAG expression (44). We also note that lowering the DNA binding activity of E47 in a DP cell line leads to the activation of CD5, CD69, and TCR expression and the downmodulation of CD4, CD8, and HSA levels. Thus, our observations indicate that E47 directly or indirectly regulates the expression of a wide variety of genes that are characteristic of thymocyte maturation.

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