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Thymocyte Selection Is Regulated by the Helix-Loop-Helix Inhibitor Protein, Id3

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Summary

E2A, HEB, E2–2, and daughterless are basic helix-loop-helix (bHLH) proteins that play key roles in multiple developmental pathways. The DNA binding activity of E2A, HEB, and E2-2 is regulated by a distinct class of inhibitor HLH proteins, the *Id* gene products. Here, we show that Id3 is required for major histocompatability (MHC) class I– and class II–restricted thymocyte positive selection. Additionally, H-Y TCR–mediated negative selection is severely perturbed in Id3 null mutant mice. Finally, we show that E2A and Id3 interact genetically to regulate thymocyte development. These observations identify the HLH inhibitory protein Id3 as an essential component required for proper thymocyte maturation.

Introduction

In the thymus, cells committed to the T lineage undergo successive stages of development to become functionally mature effector T cells. During the final stages of thymocyte maturation, CD4CD8 double-positive (DP) T cells interact with peptides associated with major histocompatibility complex (MHC) proteins to either differentiate or undergo apoptosis. If positively selected, immature DP thymocytes downregulate either CD4 or CD8 to become single-positive (SP) T cells (Jameson et al., 1994). DP thymocytes that express T cell receptors (TCRs) with high affinity for self-peptide-MHC complexes undergo programmed cell death (Robey and Folkes, 1994; von Boehmer, 1994). Developing thymocytes that do not receive TCR-mediated signals die by neglect.

During the past decade, signaling pathways originating from the TCR have been identified. However, the nuclear targets of these pathways have remained largely unknown. Recent observations have indicated that one particular transcriptional regulator, E2A, is required for proper thymocyte maturation (Bain et al., 1999b). The E2A proteins belong to a class of ubiquitously expressed basic helix-loop-helix (bHLH) transcription factors, named the E proteins. Members of this protein family include E2-2, HEB, and the *E2A* gene products E12 and E47 (Murre et al., 1989; Henthorn et al., 1990; Hu et al., 1992). E proteins are characterized by a highly conserved HLH dimerization domain and a DNA binding basic region that binds to a conserved E box motif (Murre et al., 1989). E box sites have been identified in a number of T cell–specific regulatory elements, including the CD4 and TCRβ enhancers, as well as the CD4 silencer (Takeda et al., 1990; Sawada and Littman, 1993; Duncan et al., 1996). T cell–specific E box binding complexes are largely composed of heterodimers of E2A and HEB (Sawada and Littman, 1993; Bain et al., 1997). Targeted disruption of the *HEB* or *E2A* genes leads to severe defects in thymocyte development (Zhuang et al., 1996; Bain et al., 1997). E2A-deficient mice lack subsets of γδ T cells, due in part to an impaired ability to undergo TCR V(D)J recombination (Bain et al., 1999a). E2A null mutant mice also show a partial block at the double-negative (DN) stage, prior to the onset of TCRβ chain gene rearrangement (Bain et al., 1997). In addition, E2A-deficient thymocytes exhibit a significant reduction in the proportion of CD4CD8 DP cells and a corresponding increase in CD4 and CD8 SP cells (Bain et al., 1997). HEB knockout mice exhibit a partial block in thymocyte development at the immature single-positive (ISP) cell stage. This developmental block occurs prior to the development of DP cells and results in reduced percentages of DP cells and an increased proportion of DN cells (Zhuang et al., 1996). The finding that E2A-deficient thymocytes showed increased proportions of mature SP T cells and a corresponding decrease in immature DP thymocytes suggested a role for the E2A proteins at later stages of T lymphocyte development. Recent data also indicated that proper thymocyte selection mediated by both class I and class II–restricted TCR requires the presence of E47 (Bain et al., 1999b).

In contrast to E proteins, the *Id* gene products lack a basic DNA binding domain. In vertebrates, there are four members of the *Id* gene family: *Id1*, *Id2*, *Id3*, and *Id4* (Benezra et al., 1990; Christy et al., 1991; Sun et al., 1991; Deed et al., 1992; Riechmann et al., 1994). By forming non-DNA binding heterodimeric complexes with E proteins, the *Id* gene products act as dominant-negative inhibitors of E protein activity (Benezra et al., 1990). In mice, overexpression of Id1 in B cells results in a block in B cell development at the same stage as seen in E2A-deficient mice (Sun, 1994). Additionally, retroviral infection of fetal thymic organ culture (FTOC) with Id3 leads to an inhibition of αβ T cell development and a diversion of the developmental program into the natural killer (NK) cell lineage (Heemskerk et al., 1997). Recent gene targeting experiments in mice showed that Id1 is not required for normal mammalian development (Yan et al., 1997). Id2 null mutant mice showed profound defects in peripheral lymphoid organ formation and development of NK cells (Yokota et al., 1999).

Here, we have analyzed the role of Id3 in thymocyte development. Proper maturation of SP thymocytes in class I and class II–restricted TCR transgenic mice required the presence of Id3. Additionally, the data indicate that H-Y TCR-mediated thymocyte deletion in male mice is perturbed in the absence of Id3. We propose a model in which the balance of E proteins, E2A and HEB, and their inhibitor, Id3, regulates the fate of developing thymocytes.

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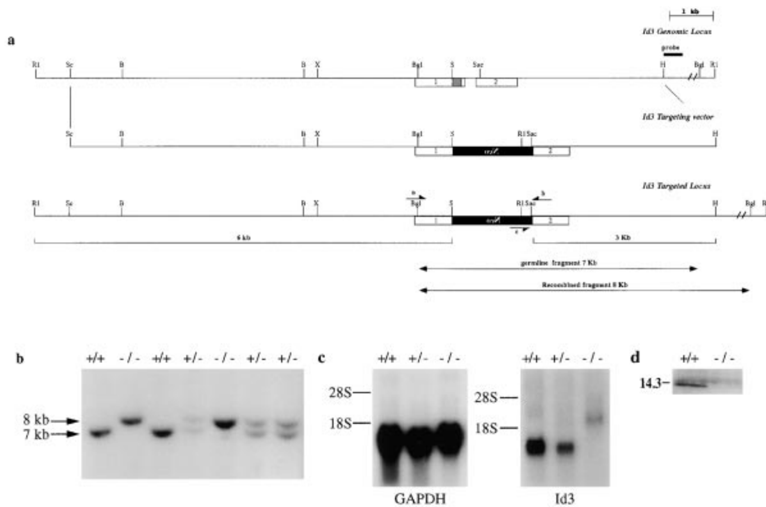

Figure 1. Generation of Id3-Deficient Mice by Gene Targeting

(A) Structure of the Id3 genomic locus, targeting vector, and targeted locus. The neomycin resistance marker gene from pMC1Neo/poly(A) replaced the HLH dimerization domain, shown as a shaded box, and is in a transcriptional orientation opposite to the *Id3* gene. The BglII digested DNA fragment sizes expected from Southern hybridization with the indicated probe are shown below. Restriction sites shown are R1, EcoR1; Sc, ScaI; B, BamHI; X, XbaI; Bgl, BglII; S, SalI; Sac, SacI; and H, HindIII. Primers used for PCR genotyping are indicated by arrows labeled "a," "b," and "c."

(B) Southern blot analysis of genomic DNA from the offspring of an Id3 heterozygous mouse cross. BglII-digested mouse tail DNA was hybridized with the radiolabeled probe shown above. Indicated are wild-type (+/+), heterozygous (+/−), and knockout mice (−/−). Arrows indicate DNA fragment sizes for the 7 kb Id3 germline and 8 kb recombined BglII fragments.

(C) Northern hybridization of thymus RNA isolated from wild-type (+/+), heterozygous (+/−), and knockout (−/−) mice. RNA was probed with Id3 (right panel) and GAPDH (left panel) probes. The positions of 28S and 18S ribosomal RNA are indicated.

(D) Western blot analysis of whole cell extract isolated from wild-type (+/+) and Id3-deficient (−/−) thymocytes. Western blots were probed with B72-1, an Id3-specific monoclonal antibody. The position of the 14.3 kDa molecular weight standard is shown.

Results

Generation of Id3-Deficient Mice

The *Id3* gene was inactivated in embryonic stem (ES) cells by gene targeting (Figure 1A). Heterozygous mutant ES cells were isolated and used to generate Id3 chimeric mice. The chimeric mice were backcrossed to C57Bl/6J mice to generate Id3 heterozygous mice. Id3 heterozygous and null mutant mice were generated from heterozygous crosses at expected transmission ratios indicating that Id3 is not essential for embryonic viability (Figure 1B). Northern blot analysis of total RNA derived from the thymus of wild-type, heterozygous, and null mutant mice showed that Id3 transcripts were reduced in heterozygous thymocytes and absent in null mutant thymocytes, confirming the inactivation of the *Id3* gene (Figure 1C). As expected, whole cell extracts derived from Id3 null mutant thymocytes lack Id3 protein (Figure 1D).

Gross examination of $Id3^{-/-}$ mice did not reveal any obvious physical abnormalities as compared to wild-type and heterozygous littermates. Since bHLH proteins have been shown to be required for proper B and T cell lineage development, we examined spleen, thymus, and bone marrow lymphocyte populations derived from Id3 null mutant mice. Normal numbers of pro-B, pre-B, im-

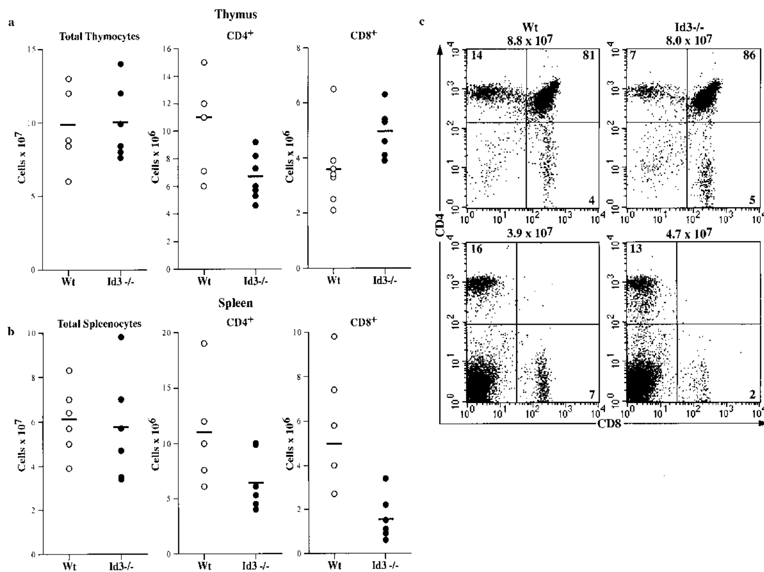

Figure 2. Impaired Thymocyte Development in Id3 Null Mutant Mice

(A) Thymoctye cell numbers from wild-type and Id3-deficient mice. Total cell numbers as well as $CD4^+$ and $CD8^+$ subpopulations from seven pairs of wild-type (open circles) and $Id3^{-/-}$ (closed circles) littermates are shown. A solid line indicates the average cell number.

(B) $CD4^+$, $CD8^+$ and total splenic T cell numbers from wild-type and Id3 null mutant mice. Open and closed circles represent wild-type and Id3-deficient mice, respectively. A solid line indicates the average cell number from six pairs of wild-type and $Id3^{-/-}$ littermates.

(C) Flow cytometric analysis of thymocytes and splenocytes from Id3 wild-type and null mutant mice stained for CD4 and CD8. Two-color flow cytometric analysis of thymocytes (top) and splenocytes (bottom) from 3-week-old wild-type (Wt) and Id3 null-mutant (−/−) littermates. T cell subpopulation percentages as well as total cell numbers are indicated. The numbers and percentages of cells shown were derived from 3- to 6-week-old mice backcrossed a minimum of five generations into the C57Bl/6J background.

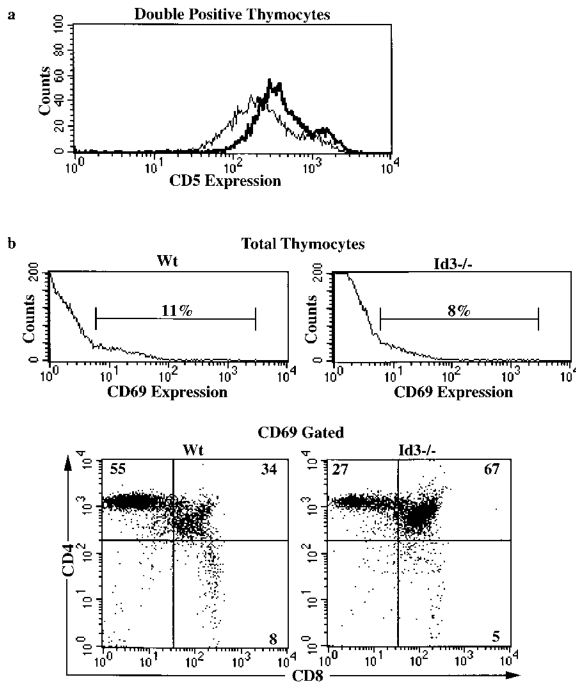

Figure 3. Abnormal DP to SP Transition in Id3-Deficient Mice

(A) *Id3*$^{-/-}$ DP thymocytes express higher levels of CD5 as compared to wild-type littermates. Three-color flow cytometric analysis of thymocytes stained for CD4, CD8, and CD5. CD5 expression was analyzed by electronically gating on DP thymocytes. Light and dark solid lines represent the analysis of wild-type and *Id3*$^{-/-}$ mice, respectively.

(B) Three-color flow cytometric analysis of CD4 and CD8 expression on CD69^{+} thymocytes. CD69^{+} thymocytes were electronically gated and analyzed for CD4 and CD8 expression. The percentage of thymocytes expressing CD69 is indicated in the top panels, and the percentage of CD69^{+} thymocytes expressing CD4 and CD8 is shown in the bottom panels.

mature-B, and mature-B cells were observed in the bone marrow, spleen, and lymph nodes of Id3-deficient mice (data not shown).

Thymocyte Development in Id3 Null Mutant Mice

In order to determine whether Id3 is required for normal T cell development, T lymphocyte populations from the thymus and spleen of Id3 null mutant and wild-type littermates were examined by flow cytometry. For this analysis, Id3-deficient mice were backcrossed a minimum of five times into the C57Bl/6J background. The average total number of thymocytes derived from *Id3*$^{-/-}$ mice was nearly identical to that of wild-type mice (Figure 2A). However, a significant decrease in both the percentage and total number of mature CD4 SP thymocytes was observed (Figures 2A and 2C). The percentage and total number of CD8 SP thymocytes was slightly increased (Figures 2A and 2C). We note that the reduction in the number of CD4 SP thymocytes observed in Id3-deficient mice was seen in both a C57Bl/6J and a 129/J; C57Bl/6J mixed background. In contrast, the number of Id3-deficient CD8 SP thymocytes in a 129/J; C57Bl/6J mixed background was slightly lower when compared to wild-type thymocytes (Rivera et al., unpublished data). In the spleen of *Id3*$^{-/-}$ mice, the proportion and number of CD4 and CD8 SP T cells was decreased when compared to wild-type littermates (Figures 2B and 2C). It is also apparent that the levels of CD4 and CD8 expression on peripheral T cells are lower in *Id3*$^{-/-}$ mice compared to wild-type littermate controls (Figure 2C). It is possible that E proteins control CD4 and CD8 expression and that a relative increase in E protein activity due to a loss of Id3 is responsible for the lower levels of CD4 and CD8 observed on *Id3*$^{-/-}$ peripheral T cells.

Early events in thymocyte selection include the upregulation of CD5 and CD69 surface expression (Bendelac et al., 1992; Yamashita et al., 1993; Brandle et al., 1994; Kearse et al., 1995). Since Id3-deficient mice showed altered proportions of SP cells, thymocyte populations were examined for the expression of CD5 and CD69. DP thymocytes isolated from *Id3*$^{-/-}$ mice showed a small but consistent increase in CD5 expression over wild-type littermates (Figure 3A). Additionally, when total thymocytes were CD69 gated, a greater percentage of DP thymocytes was observed in *Id3*$^{-/-}$ mice as compared to wild-type littermates (Figure 3B). Taken together, these observations indicate that thymocyte maturation is perturbed in Id3-deficient mice.

Thymocyte Selection in Id3 Null Mutant Mice Expressing a Class II–Restricted T Cell Receptor Transgene

To further examine the requirements for Id3 in thymocyte selection, Id3-deficient mice were crossed to AND T cell receptor (TCR) transgenic mice, which express an MHC class II–restricted TCR (Kaye et al., 1989). Development of CD4 SP thymocytes is significantly enhanced in H-2^{b} mice carrying the AND TCR transgene (Kaye et al., 1989). Thymocytes derived from wild-type, heterozygous, and null mutant mice harboring the AND TCR transgene were stained for the presence of CD4 and CD8 and analyzed by flow cytometry. Wild-type mice carrying the AND TCR transgene showed a high proportion of CD4 SP thymocytes (Figure 4A). There was a slight but significant reduction in the fraction of CD4 SP thymocytes in *Id3*$^{+/-}$; AND TCR transgenic mice as compared to wild-type littermates (Figure 4A). Strikingly, the Id3 null mutation severely impaired selection of CD4 SP thymocytes (Figure 4A). A similar reduction in CD4 SP T cells was also observed in the spleen of *Id3*$^{-/-}$; AND transgenic mice (Figure 4A). To exclude the possibility that the lack of Id3 interfered with the expression of the AND TCR transgene, thymocyte populations were examined for the expression of the Vβ3 and Vα11, AND TCR-specific Vα and Vβ chains. A significant population of Id3-deficient CD4 SP cells expressed lower levels of TCR as compared to wild-type cells (Figure 4C). These data indicate that in the absence of Id3, a significant fraction of CD4 SP cells did not upregulate TCR levels upon selection. Additionally, fewer total thymocytes from *Id3*$^{-/-}$; AND transgenic mice were CD69 positive as compared to thymocytes from *Id3*$^{+/-}$; AND TCR transgenic mice (Figure 4D). The majority of thymocytes positive for CD69 in *Id3*$^{-/-}$; AND transgenic mice were double-positive (Figure 4D). These data indicate that thymocyte maturation is blocked at the DP stage of thymocyte development, after DP thymocytes have upregulated CD69.

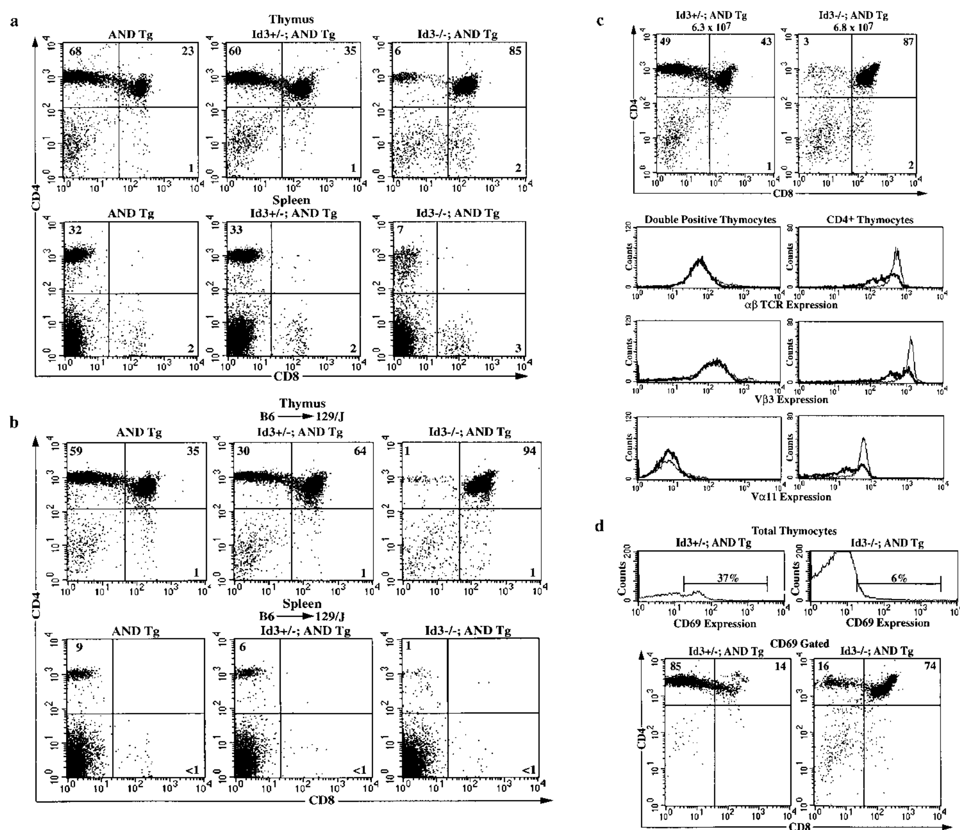

Figure 4. Id3 Is Required for Proper MHC Class II–Mediated Positive Selection

(A) Two-color flow cytometric analysis of thymocytes (top panels) and splenocytes (bottom panels) isolated from AND transgenic, *Id3*$^{+/-}$; AND transgenic and *Id3*$^{-/-}$; AND transgenic 5-week-old littermate mice stained for CD4 and CD8 expression.

(B) The positive selection defect in Id3-deficient mice is T cell intrinsic. CD4 and CD8 expression on Ly9.1 negative thymocytes (top panels) and splenocytes (bottom panels) isolated from lethally irradiated 129/J mice reconstituted with C57Bl/6J AND, *Id3*$^{+/-}$; AND and *Id3*$^{-/-}$; AND littermate fetal liver cells. Percentages of CD4$^+$ and CD8$^+$ thymocyte and splenocyte T cell populations are indicated.

(C) TCR transgene expression is reduced in CD4$^+$ thymocytes derived from *Id3*$^{-/-}$; AND transgenic mice. Three-color flow cytometric analyisis of TCR transgene expression in DP thymoyctes (left histograms) and CD4$^+$ thymocytes (right histograms) isolated from *Id3*$^{+/-}$; AND, and *Id3*$^{-/-}$; AND TCR transgenic mice. TCRαβ expression (upper histograms) as well as AND TCR transgene-specific Vα (bottom histograms), and Vβ (middle histograms) chain staining is shown. Light and dark lines represent *Id3*$^{+/-}$, and *Id3*$^{-/-}$; AND TCR transgenic mice, respectively. Genotypes, total thymocyte numbers, and CD4/CD8 expression on thymocytes used for TCR stainings are shown above histograms.

(D) Thymocyte maturation is blocked at the DP stage in *Id3*$^{-/-}$; AND TCR transgenic mice. Three-color flow cytometric analysis shows the percentage of total thymocytes expressing CD69 (histograms), as well as CD4 and CD8 expression on CD69$^+$ thymocytes. The percentage of CD69 gated thymocyte subpopulations expressing CD4 and CD8 is indicated. Thymocytes used are from the same mice used in (C); genotypes are indicated.

The abnormality in thymocyte maturation could be due to an intrinsic requirement for Id3 in thymocytes or due to a defect in cell types essential for antigen presentation, such as cortical epithelial cells. To determine whether the *Id3*$^{-/-}$; AND TCR transgenic phenotype was T cell intrinsic, fetal liver cells derived from *Id3*$^{-/-}$; AND TCR transgenic C57Bl/6J mice were introduced into lethally irradiated 129/J host mice. In chimeric mice, a severe reduction in CD4 SP cells was present in *Id3*$^{-/-}$; AND thymocytes, indicating that the Id3 mutant phenotype is T cell intrinsic (Figure 4B). Interestingly, thymocyte maturation in Id3 heterozygous mice was perturbed as well, indicating that the dosage of Id3 may influence MHC class II–restricted thymocyte maturation (Figure 4B). Taken together, these data indicate that efficient MHC class II–restricted thymocyte maturation requires the presence of Id3.

Thymocyte Selection in Id3 Null Mutant Mice Expressing a Class I–Restricted T Cell Receptor Transgene

To explore the possibility that Id3 is also required for MHC class I–restricted selection, Id3-deficient mice carrying the H-Y TCR transgene were generated. The H-Y TCR recognizes a male-specific antigen presented by class I H-2^b MHC (Kisielow et al., 1988). In male H-2^b mice, the H-Y TCR is self-reactive, resulting in a depletion of DP and SP thymocytes and a severe reduction in thymic cellularity. Conversely, in female H-2^b mice expressing the H-Y transgene, thymocyte positive selection is enhanced resulting in an increased proportion of CD8 SP T cells. Thymocyte subpopulations from female Id3-deficient mice carrying the H-Y TCR transgene were analyzed by flow cytometry. Whereas the total number of CD8 SP thymocytes was not altered in *Id3*$^{-/-}$;

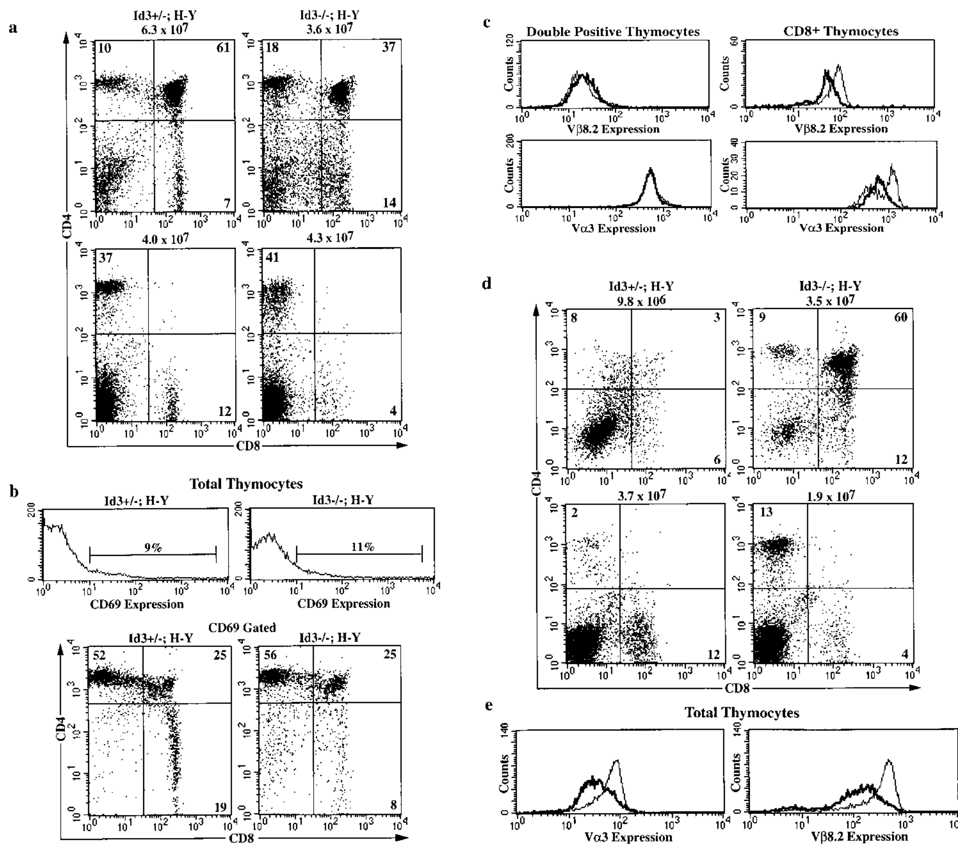

Figure 5. Id3 Is Required for Positive and Negative Selection of Thymocytes Expressing the MHC Class I–Restricted H-Y TCR Transgene

(A) Two-color flow cytometric analysis of thymocytes (top) and splenocytes (bottom) from 4-week-old female littermate mice expressing the H-Y TCR transgene. Genotypes of respective littermates and percentages of cells staining positive for CD4 and CD8 are indicated. Total cell numbers are indicated for each organ analyzed.

(B) CD69 expression on female H-Y TCR transgenic mice. Histograms show CD69 expression on total thymocytes from the same mice shown in (A). CD4 and CD8 expression on CD69 gated thymocytes is shown below. Genotypes of respective littermates as well as percentages of cells staining positive for CD4 and CD8 are indicated.

(C) CD8 SP thymocytes from Id3-deficient female H-Y TCR transgenic mice express lower levels of TCR. Three-color flow cytometric analysis was used to measure expression of H-Y TCR-specific Vα and Vβ chains on DP and CD8 SP thymocytes. Light and dark lines represent $Id3^{+/-}$, H-Y and $Id3^{-/-}$, H-Y TCR transgenic female mice, respectively. Monoclonal antibodies T3.70 and F23.1 were used for staining H-Y-specific Vα and Vβ chains, respectively. Vα-specific staining was done on the same mice shown in (A) while Vβ-specific staining is from a different pair of littermate mice.

(D) Two-color flow cytometric analysis of thymocytes (top) and splenocytes (bottom) from 3-week-old male littermate mice expressing the male-specific H-Y TCR transgene. Genotypes, cell numbers, and cells staining positive for CD4 and CD8 expression are shown.

(E) Id3-deficient male H-Y TCR transgenic mice express lower levels of the H-Y TCR transgene. Total thymoyctes from the mice shown above were stained with monoclonal antibodies T3.70 and F23.1 recognizing the Vα and Vβ chains of the male-specific H-Y TCR transgene. Light and dark solid lines represent $Id3^{+/-}$; H-Y and $Id3^{-/-}$; H-Y mice, respectively.

H-Y TCR transgenic mice, the number of CD8 SP cells that express CD69 was significantly lower (Figures 5A and 5B). Additionally, a significant fraction of Id3-deficient CD8 SP cells expressed lower levels of the H-Y TCR (Figure 5C). This altered H-Y TCR expression indicates that in the absence of Id3, a notable portion of the developing thymocytes did not upregulate TCR levels upon selection. Collectively, the data indicate that MHC class I–restricted selection is perturbed in Id3-deficient mice, albeit less severely than MHC class II–restricted selection.

To determine if the absence of Id3 also influenced H-Y TCR mediated negative selection, thymocytes isolated from $Id3^{-/-}$; H-Y male mice were analyzed by flow cytometry. As expected, $Id3^{+/-}$; H-Y TCR transgenic male mice showed a severe depletion of DP thymocytes and an overall reduction in thymic cellularity (Figure 5D). However, $Id3^{-/-}$; H-Y male littermates contained a significant DP thymocyte population (Figure 5D). Furthermore, thymic cellularity in $Id3^{-/-}$; H-Y male mice was significantly higher as compared to $Id3^{+/-}$; H-Y littermate controls (Figure 5D). $Id3^{-/-}$; H-Y TCR transgenic male splenocytes also contained a high proportion of CD4 SP T cells as compared to $Id3^{+/-}$; H-Y TCR transgenic littermates (Figure 5D). These data suggest that class II H-Y TCR mediated selection may also require the presence of Id3 (Arsov and Vukmanovic, 1999). We note that the majority of thymocytes in an $Id3^{-/-}$; H-Y male background express lower levels of the H-Y transgene (Figure 5E). It is conceivable that the Id3 deficiency

Table 1. MMTV Superantigen Deletion of CD4 and CD8 SP Cells

CD4⁺ T Cells

	Thymus			Spleen			Lymph Node		
Genotype	Vβ5.1, 5.2	Vβ11	Vβ8.1, 8.2	Vβ5.1, 5.2	Vβ11	Vβ8.1, 8.2	Vβ5.1, 5.2	Vβ11	Vβ8.1, 8.2
Id3 ±; b/b	5.3 ± 0.3	4.9 ± 0.3	17.3 ± 0.4	2.4 ± 0.0	5.2 ± 0.1	20.4 ± 2.8	2.6 ± 0.1	5.5 ± 0.3	19.5 ± 1.5
Id3 ±; b/d	1.3 ± 0.0	2.1 ± 0.1	17.9 ± 0.6	0.8 ± 0.3	1.7 ± 0.4	20.9 ± 2.0	0.5 ± 0.1	1.2 ± 0.4	20.0 ± 0.3
Id3 −/−; b/d	1.0 ± 0.4	1.7 ± 0.1	17.4 ± 0.8	1.1 ± 0.5	2.0 ± 0.8	20.4 ± 1.0	0.9 ± 0.0	1.0 ± 0.8	19.3 ± 0.3
Id3 −/−; b/d	5.7	5.1	18.1	1.9	4.4	21.2	ND	ND	ND

CD8⁺ T Cells

	Thymus			Spleen			Lymph Node		
Genotype	Vβ5.1, 5.2	Vβ11	Vβ8.1, 8.2	Vβ5.1, 5.2	Vβ11	Vβ8.1, 8.2	Vβ5.1, 5.2	Vβ11	Vβ8.1, 8.2
Id3 ±; b/b	10.8 ± 0.8	7.2 ± 0.8	16.8 ± 0.5	10.1 ± 0.7	6.7 ± 1.1	16.7 ± 0.9	10.1 ± 1.6	7.1 ± 0.2	17.2 ± 0.8
Id3 ±; b/d	0.9 ± 0.6	1.7 ± 1.0	23.1 ± 2.2	1.0 ± 0.3	2.6 ± 0.2	23.2 ± 1.6	0.9 ± 0.4	2.3 ± 0.8	24.5 ± 0.4
Id3 −/−; b/d	0.9 ± 0.7	2.0 ± 1.6	22.4 ± 0.8	1.4 ± 1.3	2.4 ± 1.9	22.3 ± 0.6	1.0 ± 0.8	2.4 ± 1.8	24.3 ± 0.1
Id3 −/−; b/b	9.8	5.7	16.0	9.1	6.3	16.5	ND	ND	ND

Percentages of thymus, spleen, and lymph node CD4⁺ and CD8⁺ T cells expressing Vβ5.1, 5.2, Vβ11 and Vβ8.1, 8.2. Percentages were calculated from lymphocytes stained with CD4 biotin, CD8 FITC, and Vβ PE antibodies followed by streptavidin cychrome secondary antibody. All (BALB/c × B6) mice were analyzed at 4 weeks of age. The average percentages and standard deviations shown were derived from two sets of littermates. The percentage of superantigen specific Vβ expressing T cells from an $Id3^{-/-}$, b/b mouse show that Vβ chain distribution is normal in $Id3^{-/-}$ mice. ND, not determined.

affects the expression of the H-Y TCR. Alternatively, *Id3*$^{-/-}$ thymocytes in a male H-Y TCR background may not efficiently exclude rearrangements of the endogenous TCR loci. Although the exact mechanism remains to be elucidated, our observations indicate that Id3 is required for H-Y TCR-mediated negative selection.

Deletion of T Cells Specific for Endogenous Superantigens Is Unaffected in Id3-Deficient Mice

In order to assess the requirement for Id3 in deletion of T cells reactive to endogenous superantigens, Id3 mutant mice were crossed with the MHC class II I-E^d mouse strain BALB/c. In (BALB/c × B6) mice, the presence of minor lymphocyte-stimulating determinants (Mls) encoded by endogenous mouse mammary tumor viruses along with expression of MHC class II I-E molecules leads to deletion of Vβ3, Vβ5, and Vβ11 bearing T cells. Vβ5⁺ and Vβ11⁺ T cells were efficiently deleted in the thymus and the periphery of Id3 null mutant (BALB/c × B6) mice, while nondeleting Vβ8 bearing T cells were not (Table 1). These data indicate that Id3 is not required for clonal deletion of T cells reactive to endogenous superantigens.

Genetic Interactions Involving Id3 and E2A in Developing Thymocytes

The data described above raise the question as to how Id3 regulates thymocyte selection. In vitro binding studies have provided ample evidence that the Id proteins function to regulate the activities of E proteins, including E2A and HEB (Benezra et al., 1990; Christy et al., 1991; Sun et al., 1991; Deed et al., 1992; Riechmann et al., 1994; Loveys et al., 1996). E2A and HEB form DNA binding heterodimers in developing thymocytes (Sawada and Littman, 1993; Bain et al., 1997). However, their DNA binding activity is downregulated in SP as compared to DP thymocytes (Bain et al., unpublished data). Furthermore, our recent observations indicate that maturation of both CD4 and CD8 SP thymocytes in E2A-deficient mice is significantly enhanced (Bain et al., 1999b). To determine if Id3 interacts genetically with either HEB or E2A, E2A/Id3 and HEB/Id3 double knockout mice were generated. Thymocyte populations from double mutant mice were analyzed by flow cytometry (Figures 6A and 6B). Due to the lethality observed in HEB-deficient mice, fetal liver cells isolated from *Id3*$^{-/-}$; *HEB*$^{-/-}$ embryos were transferred into lethally irradiated host mice in order to analyze lymphocyte populations. While the developmental defects observed in HEB knockout mice were not rescued by the absence of Id3, DP and SP thymocytes were restored to more normal proportions in E2A/Id3 double knockout mice (Figures 6A and 6B). In addition, the thymocyte numbers were significantly increased in E2A/Id3 double knockout mice as compared to *E2A*$^{-/-}$; *Id3*$^{+/-}$ mice. These observations demonstrate a genetic interaction involving the *Id3* and *E2A* genes.

These data predict that in the absence of E2A and Id3, HEB has the ability to promote thymocyte maturation. To assay for the presence of HEB DNA binding activity in *E2A*$^{-/-}$; *Id3*$^{-/-}$ thymocytes, whole cell extracts were prepared from total thymocytes and analyzed by EMSA (Figure 6C). As expected, HEB DNA binding is readily detectable in extracts derived from *Id3*$^{-/-}$; *E2A*$^{-/-}$ thymoctyes (Figure 6C). However, we note that in the absence of E2A, HEB DNA binding is significantly reduced (Figure 6C). Additionally, in the absence of Id3, HEB DNA binding is enhanced (Figure 6C).

Discussion

It is now well established that during thymocyte development, signals originating from the TCR influence the outcome of thymocyte cell fate. While the signaling pathways emanating from the TCR have been relatively well characterized, their nuclear targets have remained largely elusive. Recent data have identified the bHLH protein, E47, as an essential regulatory component of thymocyte maturation. E47/HEB DNA binding is significantly downregulated upon transition of thymocytes

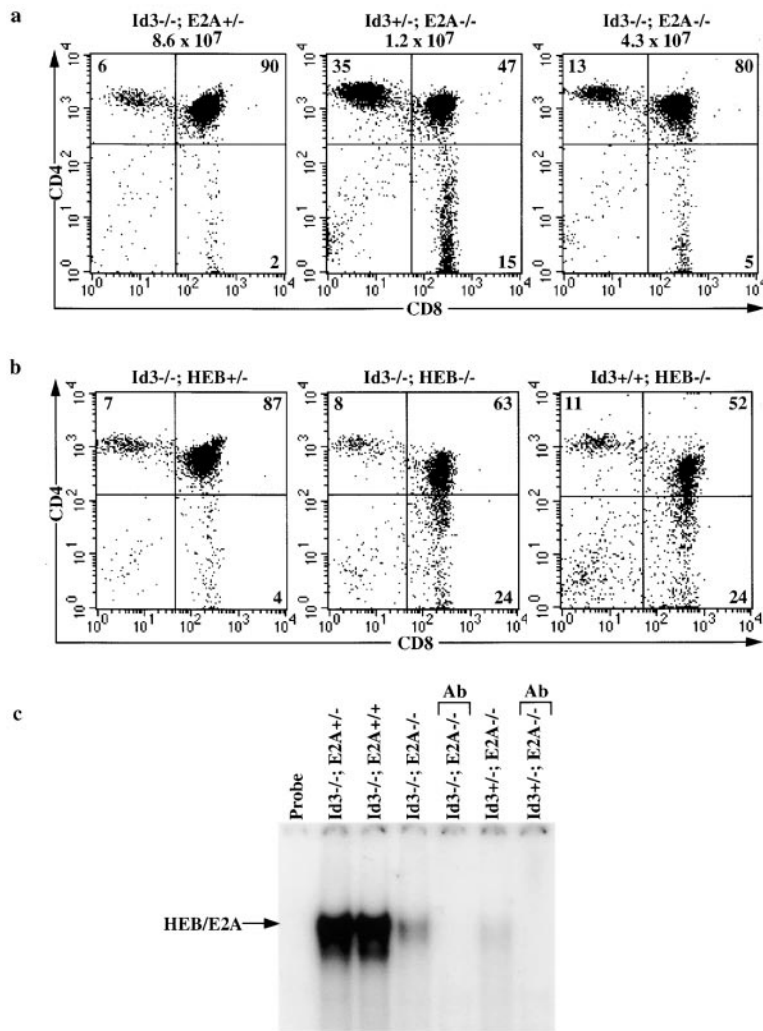

Figure 6. Genetic Interactions Involving E2A and Id3 in Developing Thymocytes

(A) Two-color flow cytometric analysis of thymocytes from *Id3*$^{-/-}$; *E2A*$^{+/-}$, *Id3*$^{+/-}$; *E2A*$^{-/-}$ and *Id3*$^{-/-}$; *E2A*$^{-/-}$ 4-week-old littermate mice. Percentages of cells staining positive for CD4 and CD8 are shown as well as total thymocyte numbers. Genotypes of respective littermate mice are indicated.

(B) Two-color flow cytometric analysis of thymocytes isolated lethally irradiated 129/J female mice reconstituted with fetal liver cells from *Id3*$^{-/-}$; *HEB*$^{+/-}$ and *Id3*$^{-/-}$; *HEB*$^{-/-}$ embryos in a C57Bl6/J background. Ly9.1 negative thymocytes staining positive for CD4 and CD8 are indicated. Thymocytes derived from an *HEB*$^{-/-}$ mouse stained with CD4 and CD8 are shown for comparison.

(C) In the absence of E2A and Id3, HEB homodimers form an E box DNA binding complex. Electrophoretic mobility shift assay (EMSA) of whole cell extracts isolated from total thymocytes. A radiolabeled μE5 E box containing oligonucleotide probe was used probe to detect E protein DNA binding. Genotypes of mice used to isolate whole cell extract and position of HEB/E2A E protein containing complexes are shown.

from the DP to the SP cell stage (Bain et al., unpublished data). Additionally, inhibiting the DNA binding activity of E47/HEB heterodimers by ectopic expression of Id3 in a DP cell line promotes in vitro differentiation (Bain et al., 1999b). These data provide compelling evidence that reducing the DNA binding activity of the E47/HEB heterodimeric complex is an important step for maturation of immature DP thymocytes to SP T cells. Consistent with these observations, increased proportions of CD4 and CD8 SP cells are present in the thymus of E47-deficient mice due to alterations in thymocyte maturation (Bain et al., 1997). Taken together, these data suggest that E protein DNA binding activity in developing thymocytes plays an important role during the final stages of thymocyte maturation.

Id3 and Thymocyte Positive Selection

Here, we show that Id3, an E protein inhibitor, influences thymocyte maturation. In the absence of Id3, class II–restricted positive selection is severely inhibited. In contrast, class I–mediated maturation is perturbed but not blocked. For the data shown here, Id3-deficient mice were backcrossed at least five times in C57Bl/6J. We note that the abnormalities in *Id3*$^{-/-}$; AND TCR–mediated maturation were observed in both a C57Bl/6J and a 129/J; C57Bl/6J mixed background. In contrast, H-Y TCR transgene-restricted maturation in a mixed background was more severe in a 129/J; C57Bl/6J mixed background, suggesting that strain differences affect the impact of Id3 on class I–mediated selection (Rivera et al., unpublished data). Collectively, the data suggest that although an Id3 deficiency affects both class I and class II–restricted maturation, class II–restricted selection is more severely perturbed when compared to class I–restricted positive selection. We note that the levels of CD4 and CD8 expressed on Id3-deficient thymocytes are quite heterogenous as compared to wild-type thymocytes and it is conceivable that Id3 regulated CD4 and CD8 gene expression by modulating E2A and HEB activity.

These observations raise the question as to how Id3 regulates positive selection. In vitro studies have indicated that Id3 interacts with the E proteins to form heterodimers that do not have intrinsic DNA binding activity (Christy et al., 1991; Deed et al., 1992, 1997, 1998; Loveys et al., 1996; Chen and Lim, 1997; Chen et al., 1997). If Id3 controls the DNA binding activity of E2A/HEB heterodimers in developing thymocytes, one would predict that Id3 levels are modulated during thymocyte differentiation. It will be interesting to determine whether Id3 is indeed regulated by signals emanating from the TCR.

Based on these observations, we propose that Id3 controls the activity of E proteins such as E2A and HEB upon TCR-mediated signals. Downregulation of E2A/HEB DNA binding activity in DP thymocytes upon TCR-mediated signaling may thus be a key step toward the differentiation of SP T lineage cells.

Id3 and Negative Selection

The observations described here show that Id3 is not required for clonal deletion of thymocytes reactive to endogenous Mtv viral superantigens. However, Id3 is required for proper negative selection of thymocytes expressing the H-Y TCR. We note that CD30-deficient thymocytes show similar abnormalities in H-Y TCR mediated negative selection, whereas appropriate deletion of thymocytes reactive to endogenous superantigens is not effected (Amakawa et al., 1996). The data raise the question as to how Id3 is involved in H-Y-mediated clonal deletion in male mice. Previous data have indicated that when overexpressed in various cell types, Id2 and Id3 have the ability to induce apoptosis (Florio et al., 1998; Norton and Atherton, 1998). Id3-induced apoptosis can be rescued by expression of E47, demonstrating the antagonistic nature of E proteins and dominant-negative Id molecules in modulating cell death and survival (Norton and Atherton, 1998). It is conceivable that Id3 similarly regulates thymocyte apoptosis during negative selection. The data also raise the question as to why Id3 is not required for superantigen-mediated negative selection. It is possible that superantigen-mediated thymocyte deletion in the medulla occurs at a developmental stage that is not sensitive to levels of Id3. We note that at this developmental stage Id2 levels are particularly high and may compensate for the loss of Id3 (Bain and Murre, unpublished data). Alternatively, it is conceivable that Id3 is required for proper differentiation of bone marrow–derived antigen-presenting cells. Since Id3 is expressed in antigen-presenting cells, this possibility cannot be excluded, and it will be interesting to examine its role in dendritic cell lineage development and function. Finally, it is plausible that the lower levels of the H-Y TCR transgene in an Id3-deficient male background allow thymocytes to escape negative selection, and further analysis will be required to address this issue.

Conclusion

Recent studies have identified bHLH proteins as important regulators of thymocyte selection. Here, we demonstrate that an HLH inhibitor functions to control thymocyte cell fate by regulating the activity of E proteins during thymocyte maturation. We note that Id3 is expressed in a wide variety of tissues, including developing B lineage cells in the bone marrow, germinal center B cells, and olfactory epithelium (Jen et al., 1997; Quong and Murre unpublished data). It will be interesting to examine whether Id3 also functions in those tissues to regulate cell fate.

Experimental Procedures

Generation of Id3 Knockout Mice

A mouse genomic 129/Sv-derived phage library was screened with a full-length mouse Id3 cDNA probe. Two independent overlapping clones were obtained that contained both Id3 exons. An approximately 9.6 kb fragment was subcloned into the pBR322 vector, the HLH dimerization domain was deleted, and a 1 kb pMC1Neo fragment was inserted. A slightly smaller ScaI fragment was then excised from the *neo* containing Id3 genomic construct, KpnI linkers were added, and the insert was ligated into the KpnI site of pBSK (Stratogene). This targeting construct was then linearized and electroporated into R1 ES cells.

Electroporation and Growth of ES Cells

129/Sv-derived R1 ES cells were cultured in DMEM high-glucose medium supplemented with 16% ES cell qualified fetal calf serum (GIBCO-BRL), 50 μM β-mercaptoethanol, 1× vitamins (GIBCO-BRL), 1× nonessential amino acids (GIBCO-BRL), and lymphocyte inhibitory factor. ES cells (1.0×10^7) were resuspended in 1 ml of media and mixed with 50 μg of linearized targeting vector. Cells were electroporated using a GIBCO-BRL gene pulser at 200 volts and a capacitance of 800 μF. After 20 min on ice, electroporated ES cells were plated onto a 15 cm tissue culture dish containing a monolayer of mitotically inactivated, *neo*-resistant, embryonic fibroblast (EF) feeder cells. Twenty-four hours post electroporation, 150 μg/ml of G418 (GIBCO-BRL) was added. After 9 days growth, G418 resistant colonies were isolated and grown in 96-well plates containing EF cells. This plate was split into two 96-well plates, and ES cells were grown to confluence. One plate was frozen in fetal calf serum containing 10% DMSO, and the other was used for isolation of genomic DNA for Southern blot analysis.

Southern and Northern Blotting

Genomic DNA was isolated from ES cells grown in 96-well plates as described. Genomic DNA was then digested and separated on a 0.8% 1× TBE gel and transferred to Nytran (Schleicher and Schull) followed by DNA cross-linking using a U.V. crosslinker (Stratogene). Wild-type and mutant Id3 alleles were identified using a HindIII–EcoRI fragment from the Id3 genomic clone. For Northern analysis, total RNA was prepared from thymus using TRIzol reagent (GIBCO-BRL). Ten micrograms of total RNA was resolved on a 1% agarose formaldehyde gel and transferred to Nytran. Northern blots were probed with GAPDH cDNA and Id3 probes. The Id3 probe was a 200 bp DNA fragment isolated by PCR with the following primers: For, 5′ CGCACTGTTTGCTGCTTTAGG 3′ and Rev, 5′ GTAGCAGTG GTTCATGTCGTC 3′.

Whole Cell Extract and Electrophoretic Mobility Shift Assay

To prepare whole cell extract from thymocytes, isolated thymocytes were collected by centrifugation at 1200 rpm at 4°C for 5 min. Cells were then washed 1× in phosphate-buffered saline (PBS) (pH 7.4). Cell pellets were frozen on dry ice, thawed, and resuspended in buffer C (20 mM HEPES [pH 7.9], .4 M NaCl, 1 mM EDTA, 1 mM EGTA including 1% NP-40, and 1 mM DTT). Cell pellets were then vortexed for 2 min and placed on ice for 10 min. Cell debris was pelleted by centrifugation, and whole cell extract supernatant was removed. Protein concentration was determined by Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA). For EMSA analysis, binding reactions were carried out with 15 μg of whole cell extract and a radiolabeled μE5, E box, oligonucleotide probe as described previously (Bain et al., 1997). For supershift analysis 382.6, a monoclonal antibody recognizing both HEB and E12 was used. 382.6 was purchased from PharMingen.

Western Blot Analysis

Twenty-six micrograms of whole cell extract protein was electrophoresed through a 4%–15% SDS polyacrylamide gradient gel and transferred to Immobilon membrane. Membranes were stained with Amido black to ensure equivalent loading of protein. Membranes were then blocked overnight at 4°C in TBST (50 mM Tris [pH 7.5], 150 mM NaCl, and .05% Tween 20) plus 5% powdered milk. A 1:250 dilution of anti-Id3 monoclonal antibody B72-1 in TBST plus 5% powdered milk was then added and incubated for 1 hr at room temperature. Blots were washed 3 × 10 min in TBST, and a 1:15,000 dilution of goat anti-mouse horseradish peroxidase (HRP)-conjugated IgG in TBST + 5% powdered milk was added for 45 min at room temperature. Western blots were then washed 4 × 5 min in

TBST and developed using the ECL plus detection system (Amersham Pharmicia Biotech). The anti-Id3 monoclonal antibody B72-1 was purchased from PharMingen.

Adoptive Transfer of Fetal Liver Cells

For adoptive transfer of Id3 AND TCR transgenic and Id3/HEB fetal liver cells, single cell suspensions of individual fetal livers from E16.5 mouse embryos in the C57Bl/6J background were prepared in Hanks' balanced salt solution containing 5% fetal calf serum and 15 mM Hepes (pH 7.0). Fetal liver cells (1×10^7) were intravenously injected into the tail vein of lethally irradiated (1000 rads) 129/J 6-week-old female recipient host mice. T cells isolated from lymphoid organs were analyzed by FACs analysis 6–8 weeks post injection. The Ly9.1 cell surface marker was used to distinguish host cells from donor cells.

TCR Transgenic Mice

H-Y and AND TCR transgenic mice were kindly provided by Dr. Stephen Hedrick. All mice analyzed were between 4 and 7 weeks of age. Id3-null-mutant mice were backcrossed five generations into the C57Bl/6J strain to generate the H-2^b background.

Mouse Genotyping

The genotype of Id3 mice was determined by Southern blotting and PCR analysis of mouse tail DNA. Two PCR primer sets were used for genotyping. One primer set flanks the *neo* insertion site and yields a 255 bp wild-type product. A second primer set detects the presence of the neomycin resistance gene by using an internal *neo* primer and a primer in the Id3 genomic locus. In the presence of *neo*, a 340 bp fragment is amplified. Primers flanking the *neo* insertion site are: For, 5′ AAGGCGCTGAGCCCGGTG 3′ and Rev, 5′CTCTGCCAG GACCACCTG 3′. The following primers detect the presence of *neo*: For, 5′ CTTGTCCTTGGAGATCAC 3′ and Rev, 5′ ACTGCATCTG CGTGTTCG 3′.

Flow Cytometric Analysis

Cells (1×10^6) were washed once in PBS and resuspended in 100 ml of FACs buffer (1× PBS, 0.1% fetal calf serum, and .01% sodium azide) containing 0.5 mg of antibody. Cells were stained in the dark on ice for 20 min. Cells were then washed 1× with 3 ml of FACs buffer. If needed, secondary reagents were added and cells were stained as before. Cells were resuspended in 1 ml of FACs buffer and analyzed on FACScan (Becton Dickinson). Antibodies used in flow cytometric analysis were against CD4, CD8, TCRαβ, CD5, CD69, Vβ5.1,5.2, Vβ8.1, 8.2, Vβ11, Vα11.1,11.2, Vβ3, Ly9.1, B220, IgM, and CD43. All antibodies were purchased from PharMingen.

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References

Amakawa, R., Hakem, A., Kundig, T., Matsuyama, T., Simard, J., Timms, E., Wakeham, A., Mittruecker, H., Griesser, H., Takimoto, H., et al. (1996). Impaired negative selection of T cells in Hodgkin's disease antigen CD30-deficient mice. Cell *84*, 551–562.

Arsov, I., and Vukmanovic, S. (1999). Dual MHC class I and class II restriction of a single T cell receptor: distinct modes of tolerance induction by two classes of autoantigens. J. Immunol. *162*, 2008–2015.

Bain, G., Engel, I., Maandag, E.R., te Riele, H.P., Voland, J., Sharp, L., Chun, J., Huey, B., Pinkel, D., and Murre, C. (1997). E2A deficiency leads to abnormalities in αβ T cell development and to the rapid development of T cell lymphomas. Mol. Cell. Biol. *17*, 4782–4791.

Bain, G., Romanow, W.J., Albers, K., Havran, W., and Murre, C. (1999a). Positive and negative regulation of V(D)J recombination by the E2A proteins. J. Exp. Med. *189*, 289–300.

Bain, G., Quong, M.W., Solow, R., Hedrick, S.M., and Murre, C. (1999b). Thymocyte maturation is regulated by the activity of the helix-loop-helix protein, E47. J. Exp. Med. *190*, 1605–1616.

Bendelac, A., Matzinger, P., Seder, R.A., Paul, W.E., and Schwartz, R.H. (1992). Activation events during thymic selection. J. Exp. Med. *175*, 731–742.

Benezra, R., Davis, R., Lockshon, D., Turner, D., and Weintraub, H. (1990). The protein Id—a negative regulator of helix-loop-helix DNA binding proteins. Cell *61*, 49–59.

Brandle, D., Muller, S., Muller, C., Hengartner, H., and Pircher, H. (1994). Regulation of Rag-1 and CD69 expression in the thymus during positive and negative selection. Eur. J. Immunol. *24*, 145–151.

Chen, B., and Lim, R. (1997). Physical and functional interactions between the transcriptional inhibitors Id3 and ITF-2B. J. Biol. Chem. *272*, 2459–2463.

Chen, B., Han, B.H., Sun, X., and Lim, R.W. (1997). Inhibition of muscle-specific gene expression by Id3: requirement of the C-terminal region of the protein for stable expression and function. Nucleic Acids Res. *25*, 423–430.

Christy, B., Sanders, L., Lau, L., Copeland, N., Jenkins, N., and Nathans, D. (1991). An Id-related helix-loop-helix protein encoded by a growth-factor inducible gene. Proc. Natl. Acad. Sci. USA *88*, 1815–1819.

Deed, R.W., Bianchi, S.M., Atherton, G.T., Johnston, D., Santibanez-Koref, M., Murphy, J.J., and Norton, J.D. (1992). An immediate early human gene encodes an Id-like helix-loop-helix protein and is regulated by protein kinase C activation in diverse cell types. Oncogene *8*, 599–607.

Deed, R.W., Hara, E., Atherton, G.T., Peters, G., and Norton, J.D. (1997). Regulation of Id3 cell cycle function by Cdk-2-dependent phosphorylation. Mol. Cell. Biol. *17*, 6815–6821.

Deed, R., Jasiok, M., and Norton, J. (1998). Lymphoid-specific expression of the Id3 gene in hematopoietic cells. J. Biol. Chem. *273*, 8278–8286.

Duncan, D., Adlam, M., and Siu, G. (1996). Asymmetric redundancy in CD4 silencer function. Immunity *4*, 301–311.

Florio, M., Hernandez, M.-C., Yang, H., Shu, H.-K., Cleveland, J., and Israel, M. (1998). Id promotes apoptosis by a novel mechanism independent of dimerization to basic helix-loop-helix factors. Mol. Cell. Biol. *18*, 5435–5444.

Heemskerk, M.H.M., Blom, B., Nolan, G., Stegmann, A.P.A., Bakker, A.Q., Weijer, K., Res, P.C.M., and Spits, H. (1997). Inhibition of T cell and promotion of natural killer cell development by the dominant negative helix loop helix factor Id3. J. Exp. Med *186*, 1597–1602.

Henthorn, P., Kiledjian, M., and Kadesch, T. (1990). Two distinct transcription factors that bind the immunoglobulin enhancer μE5/κE2 motif. Science *247*, 467–470.

Hu, J.-S., Olson, E.N., and Kingston, R.E. (1992). HEB, a helix-loop-helix protein related to E2A and ITF2 that can modulate the DNA-binding ability of myogenic regulatory factors. Mol. Cell. Biol. *12*, 1031–1042.

Jameson, S., Hogquist, K., and Bevan, M. (1994). Specificity and flexibility in thymic selection. Nature *369*, 750–752.

Jen, Y., Manova, K., and Benezra, R. (1997). Each member of the Id gene family exhibits a unique expression pattern in mouse gastrulation and neurogenesis. Dev. Dyn. *208*, 92–106.

Kaye, J., Hsu, M.-L., Sauron, M.-E., Jameson, S.C., Gascoigne, N.R.J., and Hedrick, S.M. (1989). Selective development of CD4+ T cells in transgenic mice expressing a class II MHC-restricted antigen receptor. Nature *341*, 746–749.

Kearse, K.P., Takahama, Y., Punt, J.A., Sharrow, S.O., and Singer, A. (1995). Early molecular events induced by T cell receptor (TCR) signaling in immature CD4+CD8+ thymocytes: increased synthesis of TCR-α protein is an early response to TCR signaling that compensates for TCR-α instability, improves TCR assembly, and parallels other indicators of positive selection. J. Exp. Med *181*, 193–202.

Kisielow, P., Bluthmann, J., Staerz, U.D., Steinmetz, M., and von Boehmer, H. (1988). Tolerance in T-cell receptor transgenic mice involves deletion of nonmature CD4+8+ thymocytes. Nature *333*, 742–745.

Loveys, D.A., Streiff, M.B., and Kato, G.J. (1996). E2A basic-helix-loop-helix transcription factors are negatively regulated by serum growth factors and by the Id3 protein. Nucleic Acids Res. *24*, 2813–2820.

Murre, C., McCaw, P.S., and Baltimore, D. (1989). A new DNA binding and dimerization motif in immunoglobulin enhancer binding, *daughterless*, MyoD, and myc proteins. Cell *56*, 777–783.

Norton, J.D., and Atherton, G.T. (1998). Coupling of cell growth control and apoptosis functions of Id proteins. Mol. Cell. Biol. *18*, 2371–2381.

Riechmann, V., van Cruchten, I., and Sablitzky, F. (1994). The expression pattern of Id4, a novel dominant negative helix-loop-helix protein, is distinct from Id1, Id2 and Id3. Nucl. Acids Res. *22*, 749–755.

Robey, E., and Folkes, B. (1994). Selective events in T cell development. Annu. Rev. Immunol. *12*, 675–705.

Sawada, S., and Littman, D.R. (1993). A heterodimer of HEB and an E12-related protein interacts with the CD4 enhancer and regulates its activity in T-cell lines. Mol. Cell Biol. *13*, 5620–5628.

Sun, X.-H. (1994). Constitutive expression of the Id1 gene impairs mouse B cell development. Cell *79*, 893–900.

Sun, X., Copeland, N., Jenkins, N., and Baltimore, D. (1991). Id proteins, Id1 and Id2, selectively inhibit DNA-binding by one class of helix-loop-helix proteins. Mol. Cell. Biol. *11*, 5603–5611.

Takeda, J., Cheng, A., Mauxion, F., Nelson, C.A., Newberry, R.D., Sha, W.C., Sen, R., and Loh, D.Y. (1990). Functional analysis of the murine T-cell receptor b enhancer and characteristics of its DNA-binding proteins. Mol. Cell. Biol. *10*, 5027–5035.

von Boehmer, H. (1994). Positive selection of lymphocytes. Cell *76*, 219–228.

Yamashita, I., Nagata, T., Tada, T., and Nakayama, T. (1993). CD69 cell surface expression identifies developing thymocytes which audition for T cell antigen receptor-mediated positive selection. Int. Immunol. *5*, 1139–1150.

Yan, W., Young, A.Z., Soares, V.C., Kelley, R., Benezra, R., and Zhuang, Y. (1997). High incidence of T-cell tumors in E2A-null mice and E2A/Id1 double-knockout mice. Mol. Cell. Biol. *17*, 7317–7327.

Yokota, Y., Mansouri, A., Mori, S., Sugawara, S., Adachi, S., Nishkawa, S., and Gruss, P. (1999). Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. Nature *397*, 702–706.

Zhuang, Y., Cheng, P., and Weintraub, H. (1996). B-lymphocyte development is regulated by the combined dosage of three basic helix-loop-helix genes, E2A, E2–2, and HEB. Mol. Cell. Biol. *16*, 2898–2905.