

Combined Deletion of CD8 Locus *cis*-Regulatory Elements Affects Initiation but Not Maintenance of CD8 Expression

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Summary

Developmental stage-, subset-, and lineage-specific CD8 enhancers have been identified recently by transgenic reporter analyses. Enhancer E8_{II} (CIV-4,5) is active in both immature double-positive thymocytes (DP) and mature CD8 single-positive (SP) thymocytes and T cells, whereas E8_I (CIII-1,2) directs expression only in mature cells. In mice lacking either E8_I (CIII-1,2) or E8_{II} (CIV-4,5), there was no effect on CD8 expression in DP thymocytes. However, deletion of both enhancers resulted in variegated expression of CD8, with appearance of CD4⁺CD8⁻ SP thymocytes expressing surface markers characteristic of DP thymocytes. Consequently, fewer mature CD8⁺ T cells developed from the reduced pool of DP cells. These results suggest that the initiation of CD8 expression is mediated by *cis*-regulatory elements that are distinct from any that may be involved in maintenance of expression.

Introduction

The development of T cells expressing the $\alpha\beta$ T cell receptor (TCR) specific for classical major histocompatibility complex (MHC) molecules is probably one of the best-described developmental systems in vertebrates. Several developmental stages and checkpoints have been defined based on the expression of the CD4 and CD8 coreceptor molecules. The majority of mature peripheral T cells express either CD4 or CD8. The former constitute the helper T cell lineage and express a TCR that together with CD4 is able to recognize foreign peptide presented by MHC class II molecules, while the latter display a cytotoxic phenotype and are MHC class I restricted. These single-positive (SP) cells develop in the thymus from a common CD4⁺CD8⁺ double-positive (DP) progenitor cell through positive selection of those cells that have TCRs with appropriate avidity for MHC/peptide complexes (Berg and Kang, 2001; Rothenberg, 2000). Despite the large number of studies, the molecular mechanism underlying the development of DP thymocytes toward the helper and cytotoxic T cell lineages is still largely unknown (Basson and Zamoyska, 2000; von Boehmer, 2000). It has been shown that the functional phenotype of the differentiated T cell lineage correlates with the expression of the coreceptor molecules (Chan et al., 1994; Corbella et al., 1994; Davis et al., 1993; Leung et al., 2001; Robey et al., 1994). Thus factors regulating the transcription of CD4 and CD8 may also be involved in directing the cell fate of a DP thymocyte toward the helper or cytotoxic lineage, respectively. Therefore, the identification and characterization of *cis*-regulatory elements and *trans*-acting factors involved in the transcriptional regulation of the coreceptor molecules may not only provide insight into transcriptional control mechanisms during T cell development but also may help to understand the molecular basis of lineage commitment.

During the last decade, the major *cis*-regulatory elements involved in the transcriptional regulation of CD4 have been identified. A T cell-specific enhancer located 13 kb upstream of the CD4 promoter directs expression of CD4 in both CD4⁺ helper and CD8⁺ cytotoxic T cells (Sawada and Littman, 1991), while a silencer element located in the first intron of CD4 provides subset specificity by silencing the transcription of CD4 in CD8⁺ T cells (Donda et al., 1996; Sawada et al., 1994; Siu et al., 1994). The major elements involved in the transcriptional regulation of CD8 expression have only recently been identified (for review see Ellmeier et al., 1999). Thymus-derived T cells usually express CD8 α and CD8 β heterodimers on their surface, while extrathymically derived intraepithelial lymphocytes (IEL) from the gut express only CD8 $\alpha\alpha$ homodimers (Jarry et al., 1990; Lefrancois, 1991). This partially overlapping but distinct expression pattern of the closely linked CD8 α and CD8 β genes (Gorman et al., 1988) indicates that their expression must be both coordinately and independently regulated. Hence, independent enhancers specific either for CD8 α or CD8 β may exist. By applying a transgenic reporter expression assay, several enhancers and genomic fragments involved in the regulation of CD8 expression have been identified (Ellmeier et al., 1997, 1998; Hostert et al., 1997b, 1998; Zhang et al., 1998). Four different genomic fragments isolated from the CD8 locus individually were able to direct expression in a developmental stage-, subset-, and lineage-specific mode (Figure 1A). One enhancer, designated E8_I (CIII-1,2), had activity only in mature CD8⁺ T cells, while another one, E8_{III} (CIV-3), directed expression only in immature DP thymocytes. Enhancer E8_{II} (CIV-4,5) directed expression both in DP thymocytes and CD8⁺ T cells, and an additional *cis*-regulatory element, E8_{IV} (CIV-1,2), also showed low activity in CD4⁺ T cells in addition to CD8⁺ T cells. While all the *cis*-acting elements were active in thymic-derived T cells (at least at certain developmental stages), only E8_I (CIII-1,2) directed expression of CD8 in extrathymically derived IEL (Ellmeier et al., 1997). Taken together, these studies indicate a complex regulatory network to achieve lineage-specific regulation of CD8 α and CD8 β gene expression during T cell development. The results

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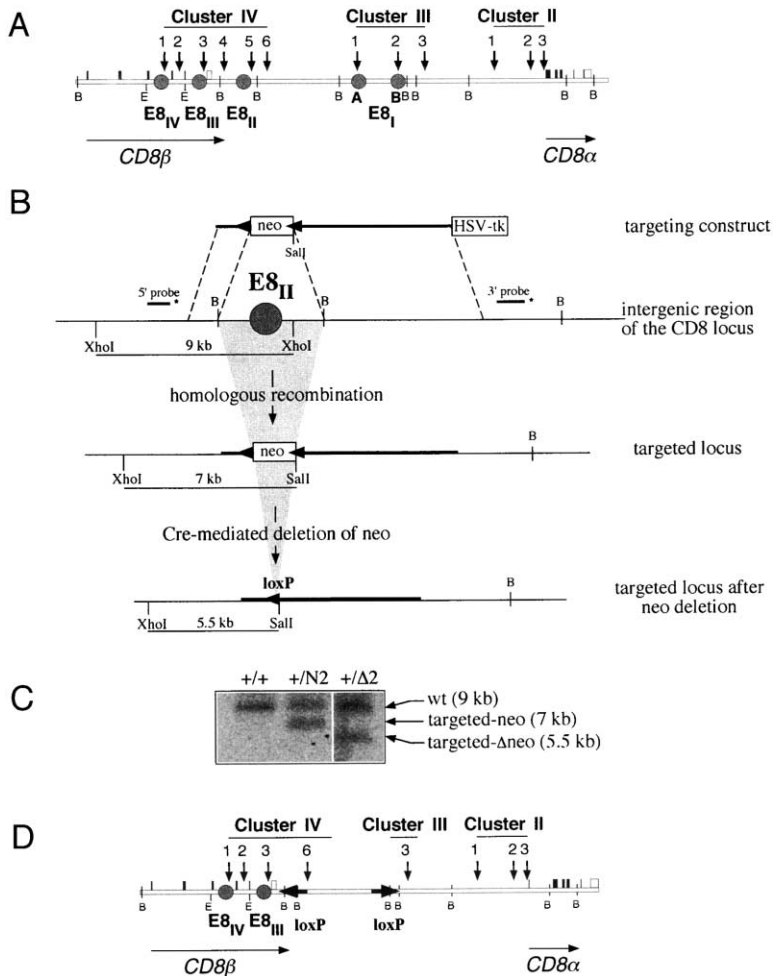


Figure 1. Targeting Strategy for the Deletion of the Enhancer E8_{II} (CIV-4,5)

(A) Schematic map of the *CD8* locus with clusters of DNase I hypersensitivity sites. Horizontal arrows indicate the transcriptional orientation of the *CD8β* and *CD8α* genes. Vertical arrows indicate the localization of DNase I hypersensitivity sites that constitute clusters II, III, and IV (Hostert et al., 1997a). Shaded circles indicate enhancers E8_I (CIII-1,2) (divided into subregions E8_{IA} and E8_{IB}), E8_{II} (CIV-4,5), E8_{III} (CIV-3), and E8_{IV} (CIV-1,2), as shown previously (Ellmeier et al., 1998). All BamHI (B), but only relevant EcoRI (E) sites are shown.

(B) Schematic map of the targeting construct (top), the *CD8* locus before and after homologous recombination (middle), and the genomic locus after Cre recombinase-mediated deletion of the neomycin resistance gene (bottom). Only restriction sites important for the targeting strategy are shown. The horizontal thick black line (in the top, lower middle, and bottom) indicates the region of homology between the targeting construct and the endogenous locus. The bars with asterisks (in the upper middle) represent the 5' and 3' probes used for Southern blotting. Horizontal bars with numbers (indicating the size in kb) represent the expected genomic fragments after digestion with the appropriate restriction enzymes (XhoI and Sall for the 5' targeted region).

(C) Southern blot of XhoI/Sall-digested DNA isolated from a wild-type ES cell clone (+/+), from ES cell clones after homologous recombination (+/N2), and from an ES cell clone after Cre recombinase-mediated deletion of the neomycin resistance cassette (+/Δ2). The origin of the detected fragments is indicated in (B).

(D) Map of the *CD8* locus showing the remaining enhancers and DNase I hypersensitivity sites after the deletion of enhancers E8_I (CIII-1,2) and E8_{II} (CIV-4,5). It was confirmed by Southern blotting that no additional alteration, such as inversion of the genomic fragment flanked by the two remaining loxP sites, occurred at the *CD8* locus (data not shown).

from the transgenic reporter expression assays raise the question of why so many different *cis*-acting elements are required for the regulation of CD8 expression. To investigate whether different elements have unique regulatory functions, we and others have begun a systematic deletional analysis of enhancers in the mouse germ line (Ellmeier et al., 1998; Hostert et al., 1998). The generation of E8_I (CIII-1,2)-deficient ($\Delta 1/\Delta 1$) mice revealed that E8_I (CIII-1,2) is the major *cis*-regulatory element that directs CD8 α expression in extrathymically derived IELs. In contrast, thymic-derived T cell lineages from these mutant mice displayed a normal expression pattern of CD8, indicating that other elements are able to compensate for loss of E8_I (CIII-1,2).

In this study, we have focused on enhancer E8_{II} (CIV-4,5), which was shown to have activity in DP and CD8 SP thymocytes and in mature CD8⁺ T cells (Ellmeier et al., 1998). We wished to determine whether the E8_{II} (CIV-4,5) activity observed in transgenic mice reflects a unique and nonredundant function of this enhancer in the regulation of *CD8α* and *CD8β* gene expression during the development of thymic-derived T cells. We found that E8_{II} (CIV-4,5)-deficient ($\Delta 2/\Delta 2$) mice had normal ex-

pression of CD8, both in thymus-derived T cells and in IELs, and had no thymic developmental alterations. This indicated that other regulatory elements at the *CD8* locus are sufficient to direct normal expression of CD8 in the absence of E8_{II} (CIV-4,5). Since in transgenic mice both E8_I (CIII-1,2) and E8_{II} (CIV-4,5) direct expression in mature CD8⁺ T cells, we generated E8_I (CIII-1,2)/E8_{II} (CIV-4,5) double-deficient mice ($\Delta 1\Delta 2/\Delta 1\Delta 2$ mice) to test whether these two enhancers are able to compensate for each other. In contrast to individual deletions, deletion of both enhancers had a major effect on the expression of CD8 during thymocyte development but not in mature CD8 lineage cells. A population of "CD8-negative" SP thymocytes appeared that was indistinguishable from DP thymocytes by analysis of other surface markers and by functional phenotype. The emergence of this subset of cells indicated that CD8 expression is variegated in DP thymocytes of $\Delta 1\Delta 2/\Delta 1\Delta 2$ mice. Surprisingly, expression of CD8 on DP cells from $\Delta 1\Delta 2/\Delta 1\Delta 2$ mice was at a level similar to that from wild-type littermates. These results reveal a previously unexpected function of two distinct enhancer elements, suggest that additional enhancers contribute to the initiation

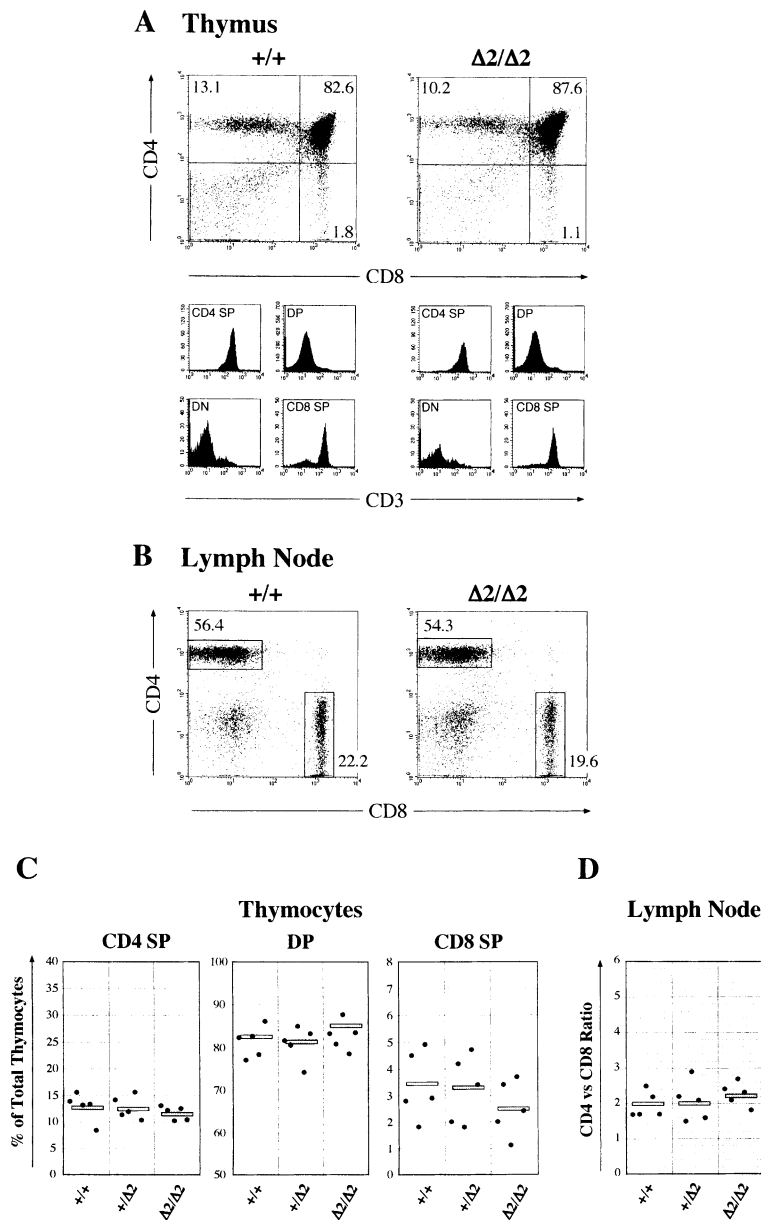


Figure 2. Normal T Cell Development and CD8 Expression in $\Delta 2/\Delta 2$ Mice

(A) Three-color flow cytometric analysis of CD4, CD8, and CD3 expression on thymocytes isolated from $+/+$ and $\Delta 2/\Delta 2$ littermates. Upper panel: numbers in the dot plot quadrants indicate the percentage of the corresponding thymocyte subpopulations (of total gated thymocytes). Lower panel: individual histograms showing CD3 expression on gated thymocyte populations (gated populations are indicated in the upper left of each histogram).

(B) Lymph node T cells were isolated from $+/+$ and $\Delta 2/\Delta 2$ littermates and analyzed by two-color flow cytometry for expression of CD4 and CD8. Numbers next to the gates indicate the percentage of the corresponding lymph node cell population (of total gated cells).

(C) Diagram indicating the percentage of CD4 single-positive (SP, left panel), double-positive (DP, middle panel), and CD8 SP (right panel) thymocytes in wild-type ($+/+$), heterozygous ($+/\Delta 2$), and homozygous ($\Delta 2/\Delta 2$) knockout mice.

(D) Diagram showing the CD4 to CD8 ratio of lymph node T cells isolated from mice of the indicated genotypes. In (C) and (D), each circle represents one mouse. Horizontal bars indicate average value of each genotype.

of CD8 expression in DP thymocytes, and indicate that an active state of CD8 gene expression is epigenetically maintained during development of DP thymocytes.

Results

Generation of $\Delta 2/\Delta 2$ Mice

$E8_{II}$ (CIV-4,5) is located on a 4.3 kb genomic BamHI fragment (Figure 1A) downstream of the *CD8 β* gene (Ellmeier et al., 1998). This fragment was deleted by homologous recombination in E14.1 ES cells (Figures 1B and 1C), and several targeted clones were isolated (designated $+/N2$). Heterozygous $+/N2$ ES cells were then transiently transfected with Cre recombinase to delete the neomycin selection cassette, which has the potential to interfere with the regulation of neighboring enhancer and promoter elements (Sleckman et al., 1996). This gen-

erated ES cells in which only a single loxP site remained instead of $E8_{II}$ (CIV-4,5). Heterozygous $+/\Delta 2$ ES cell clones were then selected for injection into C57Bl/6 blastocysts, and chimeric mice obtained from $+/\Delta 2$ ES cells were backcrossed to C57Bl/6 mice for germline transmission. Heterozygous offspring were intercrossed to obtain $+/+$, $+/\Delta 2$, and $\Delta 2/\Delta 2$ mice. Mice were also generated from $+/N2$ ES cells, and $N2/N2$ mice showed the same phenotype as $\Delta 2/\Delta 2$ mice (data not shown).

Unaltered T Cell Development and CD8 Expression in $\Delta 2/\Delta 2$ Mice

The 4.3 kb genomic fragment containing $E8_{II}$ (CIV-4,5) enhancer activity is able to direct the expression of a transgenic hCD2 reporter gene both in DP thymocytes and mature CD8 T cells (Ellmeier et al., 1998). However, the deletion of $E8_{II}$ (CIV-4,5) had no effect on the thymic

Table 1. Relative Expression of CD8 and CD4 in Enhancer-Deficient Mice

Genotype	Thymocyte Subset				
	Coreceptor	DP	CD8 SP	CD4 SP	Lymph Node
WT	CD8	100	100		100
(n = 5)	CD4			100	100
$\Delta 2/\Delta 2$	CD8	94 ± 18	89 ± 23		102 ± 19
(n = 7) ^a	CD4			94 ± 20	107 ± 15
$\Delta 1\Delta 2/\Delta 1\Delta 2$	CD8	80 ± 13	75 ± 14		68 ± 5
(n = 7)	CD4			101 ± 17	99 ± 11
$\Delta 1/\Delta 1$ (n = 6) ^b	CD8	98 ± 10	73 ± 11		98 ± 11

Numbers indicate the percentage of CD8 or CD4 expression levels (measured as ratios of mean fluorescence) of homozygous enhancer-deficient mice relative to wild-type mice in thymic subpopulations and in lymph node T cells.

^an = 5 for lymph node expression levels.

^bNumbers were previously reported in Ellmeier et al., 1998.

(Figure 2A) or peripheral expression of CD8 (Figure 2B and Table 1). Furthermore, T cell development was normal and there was no difference in the relative abundance of the different T cell subsets compared to wild-type mice (Figure 2C and data not shown). In addition, the expression of CD8 α homodimers on IEL was similar in wild-type and $\Delta 2/\Delta 2$ mice (Figure 6B).

Generation of $\Delta 1\Delta 2/\Delta 1\Delta 2$ Mice

The results from the analysis of $\Delta 2/\Delta 2$ mice clearly indicated that other regulatory elements were able to compensate for loss of E8_{II} (CIV-4,5) enhancer activity. Other enhancers such as the mature CD8 enhancer E8_I (CIII-1,2) or the DP-specific enhancer E8_{III} (CIV-3) are able to direct expression in similar T cell subsets as E8_{II} (CIV-4,5) does (Ellmeier et al., 1999). Thus, it is possible that E8_I (CIII-1,2) is able to compensate for loss of E8_{II} (CIV-4,5) in mature CD8 T cells, while E8_{III} (CIV-3) might compensate for the loss of E8_{II} (CIV-4,5) in DP thymocytes. In fact, the normal expression of CD8 in thymus-derived T cell lineages in $\Delta 1/\Delta 1$ mice (Ellmeier et al., 1998; Hostert et al., 1998) indicates that elements exist which compensate for loss of E8_I (CIII-1,2) as well. To test whether E8_{II} (CIV-4,5) and E8_I (CIII-1,2) have redundant functions in the regulation of CD8 α and CD8 β gene expression, we generated mice lacking both enhancers. Therefore, $+\Delta 2$ ES cells were transfected with the E8_I (CIII-1,2) targeting construct (Ellmeier et al., 1998), and E8_I (CIII-1,2)-targeted $+\Delta 2$ ES cells were subsequently screened by Southern blotting to identify ES cells that had both enhancers deleted at the same CD8 locus allele. Heterozygous $+/N1\Delta 2$ ES cells were then injected into C57Bl/6 blastocysts, and subsequently $+/N1\Delta 2$ mice were obtained. To delete the neomycin expression cassette, $+/N1\Delta 2$ mice were crossed with transgenic mice expressing Cre recombinase under the control of the CMV promoter (White et al., 1997). Heterozygous $+/N1\Delta 2$ mice were then interbred to obtain $+/+$, $+/N1\Delta 2$, and $\Delta 1\Delta 2/\Delta 1\Delta 2$ mice [see Figure 1D for the map of the CD8 locus after E8_I (CIII-1,2) and E8_{II} (CIV-4,5) deletion]. Expression of CD8 was also analyzed in $N1\Delta 2/N1\Delta 2$ mice, and they had the same phenotype as $\Delta 1\Delta 2/\Delta 1\Delta 2$ mice (data not shown).

Altered T Cell Development and CD8 Expression in $\Delta 1\Delta 2/\Delta 1\Delta 2$ Mice

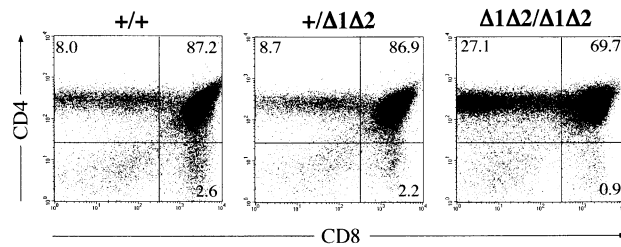
Deletion of E8_I (CIII-1,2) (Ellmeier et al., 1998; Hostert et al., 1998) or E8_{II} (CIV-4,5) individually had no effect on

T cell development or on the expression of CD8 in thymus-derived T cells. In contrast, thymocytes from double-deficient mice displayed a dramatic change in the CD4 versus CD8 expression profile (Figures 3A and 3C). An increase in CD4 SP thymocytes was observed (from 11% to 29%), while the percentage of DP thymocytes decreased (from 84% to 65%). In addition, the percentage (from 4.5% to 1.8%; Figure 3C) and also the absolute numbers (from $2.8 \pm 0.2 \times 10^6$ in $+/N1\Delta 2$ mice to $1.9 \pm 0.2 \times 10^6$ in $\Delta 1\Delta 2/\Delta 1\Delta 2$ littermates; n = 3 and 4, respectively) of CD8 SP thymocytes in the thymus of $\Delta 1\Delta 2/\Delta 1\Delta 2$ mice were decreased. However, the total number of thymocytes in $+/N1\Delta 2$ and $\Delta 1\Delta 2/\Delta 1\Delta 2$ mice was not different from wild-type littermates (data not shown). Changes in the percentage of CD4⁺ versus CD8⁺ T cells were also observed in the periphery (Figure 3B). $\Delta 1\Delta 2/\Delta 1\Delta 2$ mice had fewer CD8⁺ T cells than their wild-type littermates, indicated by an increase in the CD4/CD8 ratio in secondary lymphoid organs such as lymph nodes (from 1.7 to 3.6; Figures 3B and 3D) and spleen (data not shown). Lymph node T cells that still expressed CD8 had approximately 70% of wild-type CD8 expression levels on their surface (Table 1). Furthermore, all CD8-expressing thymocytes and T cells expressed both CD8 α and CD8 β (Figure 4E and data not shown).

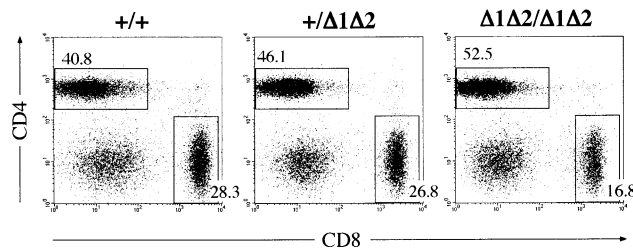
“CD8-Negative” SP Thymocytes in $\Delta 1\Delta 2/\Delta 1\Delta 2$ Mice

The increase in the percentage of CD4 SP thymocytes could be explained by at least three mechanisms. It could be caused either by a loss of CD8 expression on DP thymocytes, by an increase in positive selection of CD4 lineage cells, or even by a developmental switch from the CD8 to CD4 T cell lineage caused by alterations in the expression of CD8. To investigate whether one of these mechanisms was responsible for the altered thymic profile in $\Delta 1\Delta 2/\Delta 1\Delta 2$ mice, we determined the developmental stage of the CD4 SP cells by examining the expression of CD3, HSA, CD69, and CD5. As previously shown (Bendelac et al., 1992; Swat et al., 1993; van Meerwijk and Germain, 1993; Yamashita et al., 1993), wild-type CD4 SP thymocytes have a CD3^{hi}, HSA^{lo}, CD5^{hi}, and CD69^{+/+} surface phenotype (Figure 4B), while wild-type DP thymocytes are CD3^{lo/med}, HSA^{hi}, CD5^{med}, and CD69^{-/lo}. CD4 SP thymocytes from $\Delta 1\Delta 2/\Delta 1\Delta 2$ mice, however, displayed a bimodal expression pattern of these surface molecules (Figure 4B), suggesting the presence of two distinct subsets. In addition to the ma-

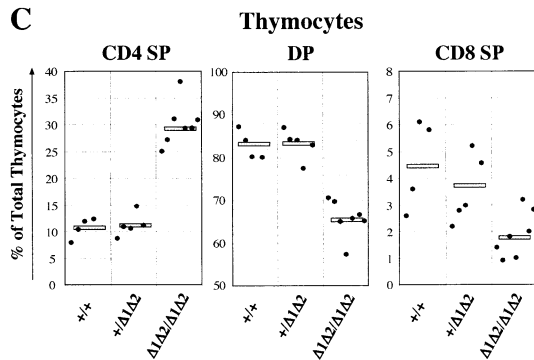
A Thymus



B Lymph Node



C



D Lymph Node

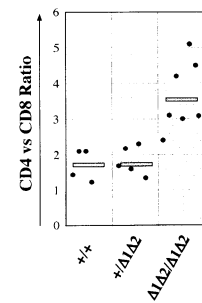


Figure 3. Altered CD8 Expression and Reduced Numbers of CD8 SP Thymocytes and CD8⁺ T Cells in $\Delta 1\Delta 2/\Delta 1\Delta 2$ Mice

(A) Flow cytometric analysis of CD4 and CD8 expression on thymocytes isolated from mice of the same litter with the indicated genotype. Numbers in the dot plot quadrants indicate the percentage of the corresponding thymic subpopulations (of total gated thymocytes). (B) Lymph node T cells isolated from mice of the indicated genotype were analyzed by two-color flow cytometric analysis for the expression of CD4 and CD8. Gates show the area of either CD4⁺ or CD8⁺ T cells. Numbers next to the gates indicate the percentage of the corresponding T cell subset.

(C) Diagram indicating the percentage of CD4 single-positive (SP, left panel), double-positive (DP, middle panel), and CD8 SP (right panel) thymocytes in wild-type (+/+), heterozygous (+/ $\Delta 1\Delta 2$), and homozygous ($\Delta 1\Delta 2/\Delta 1\Delta 2$) knockout mice.

(D) Diagram showing the CD4 to CD8 ratio of lymph node T cells isolated from mice of the indicated genotypes. In (C) and (D), each circle represents one mouse. Horizontal bars indicate average value of each genotype.

ture CD4 SP thymocyte subset, a population of CD4 SP cells that were CD3^{lo}, HSA^{hi}, CD5^{med}, and CD69^{neg} was detected. Thus, the surface phenotype of these additional CD4 SP thymocytes is similar to DP thymocytes. To test whether TCR engagement in the absence of both enhancers at the onset of positive selection caused the downmodulation of CD8 on DP thymocytes, $\Delta 1\Delta 2/\Delta 1\Delta 2$ mice were intercrossed with TCR α -deficient mice (Mombaerts et al., 1992; Philpott et al., 1992). As shown in Figure 4C, “CD8-negative” SP thymocytes were present in $\Delta 1\Delta 2/\Delta 1\Delta 2$ -TCR α null mice. This indicates that the alterations in CD8 expression occurred at early stages (i.e., before positive selection) of T cell development.

To functionally test whether the “CD8-negative” SP thymocytes are indeed DP thymocytes that do not express CD8, their sensitivity toward glucocorticoids was determined. Dexamethasone injection caused the disappearance of both DP and “CD8-negative” SP thymocytes after 48 hr but had no effect on CD4 SP cells in control mice (Figure 4D). In addition, “CD8-negative” SP thymocytes and DP thymocytes from mutant mice displayed similar proliferative profiles in short-term BrdU labeling experiments (data not shown).

We also wished to determine whether “CD8-negative” SP thymocytes express CD8 β (which requires CD8 α for surface expression). To test for the expression of CD8 β ,

we took advantage of the existence of two allelic forms of CD8 β (and also CD8 α) that can be distinguished from each other using specific antibodies. AKR.1 mice express the CD8 β .1 isoform, while C57Bl/6 (or 129/Sv) mice express CD8 β .2. Therefore, AKR.1 mice were crossed with +/ $\Delta 1\Delta 2$ mice (which are CD8 β .2), and subsequently the F1 generation (either +^{AKR.1}/^{C57Bl/6} or +^{AKR.1}/ $\Delta 1\Delta 2$ ^{C57Bl/6}) was analyzed for the expression of the various CD8 β alleles. As shown in Figure 4E, a population of “CD8 β .2-negative” SP thymocytes was detected only in +^{AKR.1}/ $\Delta 1\Delta 2$ ^{C57Bl/6} mice, thus showing that “CD8-negative” SP thymocytes express neither CD8 α nor CD8 β .

Variegated Expression of CD8 in $\Delta 1\Delta 2/\Delta 1\Delta 2$ DP Thymocytes

Following β selection and proliferation of double-negative stage 3 (DN3) thymocytes, CD8 and CD4 are expressed and the cells progress to the DP stage. To follow the development of “CD8-negative” SP thymocytes and DP thymocytes in $\Delta 1\Delta 2/\Delta 1\Delta 2$ mice, fetal thymic organ culture (FTOC) experiments were performed. E14.5 thymic lobes from +/ $\Delta 1\Delta 2$ and $\Delta 1\Delta 2/\Delta 1\Delta 2$ mice were isolated and cultured, and the appearance of cells expressing CD4 and CD8 was monitored over a period of several days. The vast majority of +/ $\Delta 1\Delta 2$ and $\Delta 1\Delta 2/\Delta 1\Delta 2$ thymocytes

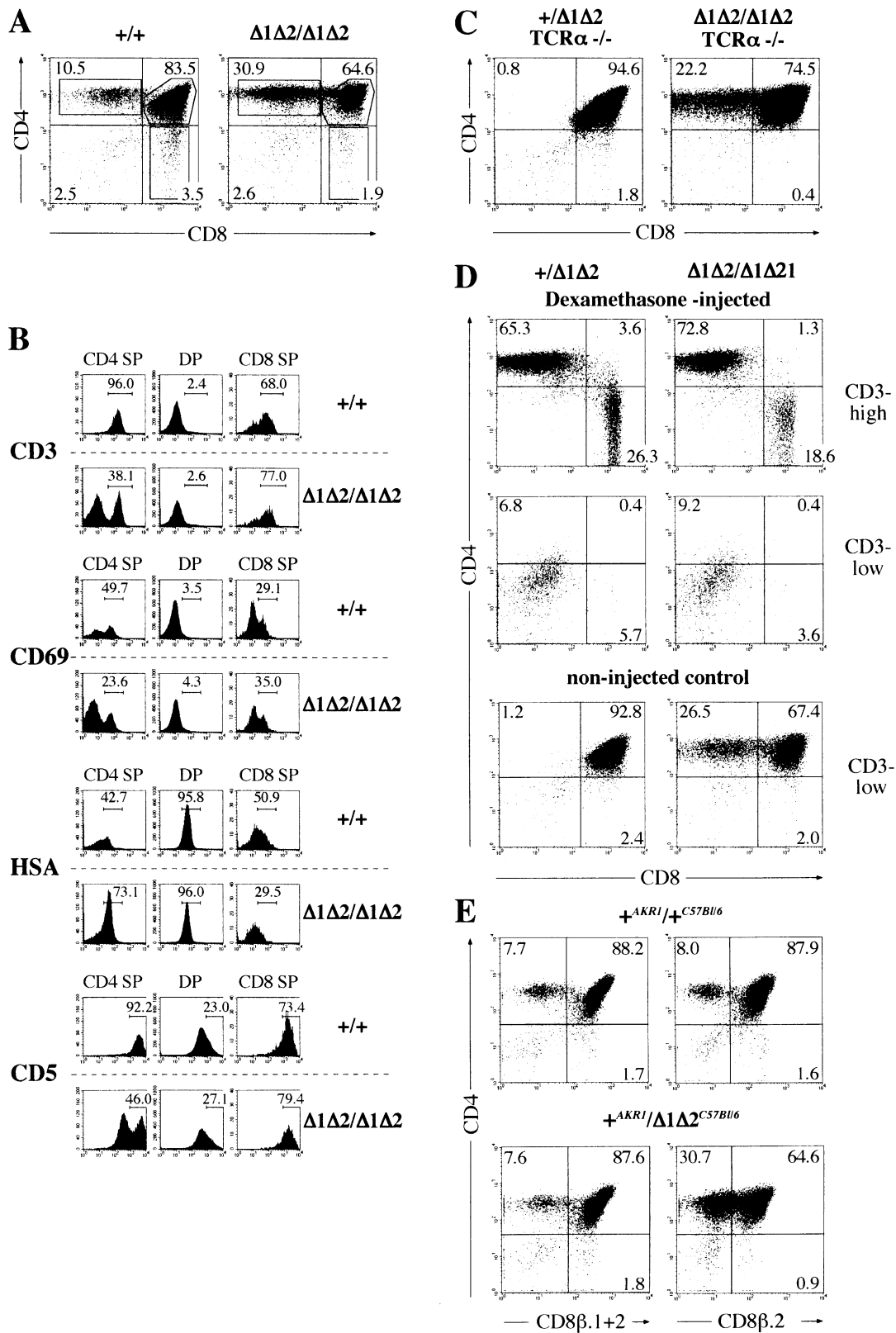


Figure 4. "CD8-Negative" SP Thymocytes in $\Delta 1\Delta 2/\Delta 1\Delta 2$ Mice

(A) Three-color flow cytometric analysis of thymocytes isolated from $+/+$ or $\Delta 1\Delta 2/\Delta 1\Delta 2$ littermates. Numbers in the CD4 versus CD8 dot plot quadrants indicate the percentage of the corresponding thymocyte subpopulations (of total gated thymocytes). Regions indicate gating areas for the histograms shown in (B).

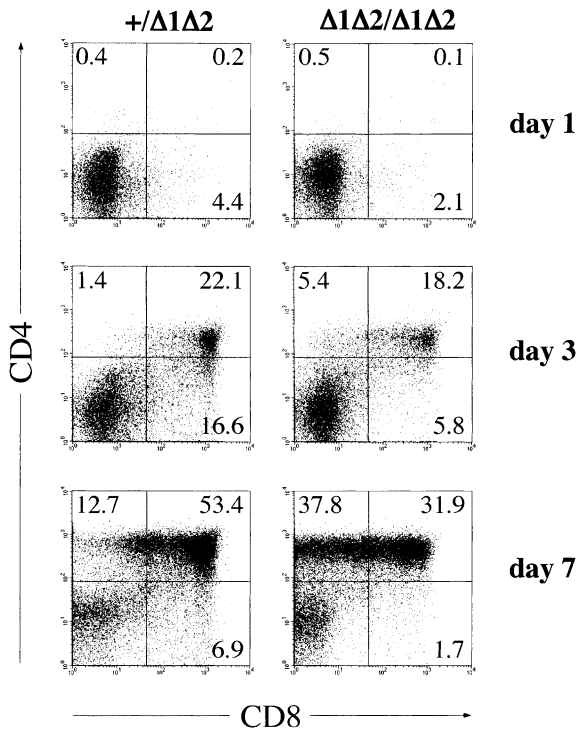


Figure 5. Monitoring the Appearance of “CD8-Negative” SP Thymocytes

Flow cytometric analysis of fetal thymic organ cultures. Thymic lobes (E14.5) were isolated and cultured *in vitro* for the indicated time periods. Dot plots show the expression of CD4 and CD8 coreceptor molecules. Numbers in the dot plot quadrants indicate the percentage of the corresponding thymic subpopulations (of total gated thymocytes).

mocytes were still at the DN stage after 1 day in culture (Figure 5), although some immature CD8 SP (ISP) thymocytes were detected. After 3 days, a high percentage of ISP and DP thymocytes but only few CD4 SP cells were present in the +Δ1Δ2 culture. In the Δ1Δ2/Δ1Δ2 culture, DP thymocytes and ISP were also present. However, there was an increase in the percentage of “CD8-negative” SP thymocytes, and therefore the ratio of CD4 SP to DP cells was much higher than in the +Δ1Δ2 culture. In addition, a decrease in the percentage of immature CD8 SP Δ1Δ2/Δ1Δ2 thymocytes was observed. The emergence of “CD8-negative” SP thymocytes in Δ1Δ2/Δ1Δ2 cultures was even more pronounced after 7 days.

Thus, “CD8-negative” SP thymocytes and DP cells in Δ1Δ2/Δ1Δ2 mice appear to develop simultaneously.

E8_I (CIII-1,2) and E8_{II} (CIV-4,5) Direct CD8αα Homodimer Expression in TCRγδ IEL

It has previously been shown, both by transgenic reporter expression and targeted deletion in ES cells, that E8_I (CIII-1,2) is the major enhancer involved in the regulation of CD8α expression on IEL (Ellmeier et al., 1998; Hostert et al., 1998). However, a subset of IEL in Δ1/Δ1 mice still shows low-level expression of CD8α. Thus, other enhancers must be involved in the regulation of CD8α in IEL. However, as mentioned above and shown in Figure 6B, E8_{II} (CIV-4,5)-deficient IEL displayed normal levels of CD8α. In sharp contrast, double-deficient mice essentially lost CD8α expression on IEL of the TCRγδ lineage (Figure 6A). A reduction of CD8α expression in Δ1Δ2/Δ1Δ2 mice compared to Δ1/Δ1 mice was also observed on TCRαβ-positive IEL (data not shown).

Discussion

Several enhancers within the *CD8* locus have been shown to direct expression of reporter genes in transgenic mice in a developmental stage-, subset-, and lineage-specific fashion. Among these, E8_I (CIII-1,2) is active only in mature CD8 T cells and IELs, while E8_{II} (CIV-4,5) is active both in mature CD8 T cells and DP thymocytes (Ellmeier et al., 1999). To understand the function and potential interactions of the different enhancers at the endogenous locus in more detail, we have employed gene targeting to delete enhancers in the mouse germ line. In this study, we generated E8_{II} (CIV-4,5) single (Δ2/Δ2) or E8_I (CIII-1,2)/E8_{II} (CIV-4,5) double-deficient (Δ1Δ2/Δ1Δ2) mice and showed that these two enhancers function together in an unexpected manner in the establishment of CD8 expression in immature thymocytes. Single or double mutations had little effect on CD8 expression in mature CD8 lineage T cells, and single mutations also had no effect on expression of CD8 in thymocytes. However, deletion of both enhancers led to the appearance of “CD8-negative” SP thymocytes that, by several criteria, were shown to be DP thymocytes that do not express CD8. The concurrent appearance of “CD8-negative” SP thymocytes and DP cells is consistent with variegation of expression of CD8 in Δ1Δ2/Δ1Δ2 DP thymocytes, which suggests that precursor cells in mutant mice undergo stochastic establishment or loss of *CD8* gene expression. As a consequence,

(B) Individual histograms showing expression of CD3, CD69, HSA, and CD5 (from top to bottom). Histograms above the dotted line show expression in wild-type (+/+) thymocytes, while the lower rows of histograms indicate expression in Δ1Δ2/Δ1Δ2 littermates. The thymocyte subsets that were analyzed are indicated on the top of the histograms. Numbers in the histograms show the percentage of cells within the indicated regions.

(C) Two-color flow cytometric analysis of CD4 and CD8 expression on thymocytes isolated from either +Δ1Δ2 or Δ1Δ2/Δ1Δ2 TCRα-deficient littermates. Numbers in the dot plot quadrants indicate the percentage of the corresponding thymocyte subpopulations (of total gated thymocytes).

(D) Three-color flow cytometric analysis of thymocytes isolated from +Δ1Δ2 or Δ1Δ2/Δ1Δ2 mice 48 hr after i.p. injection of 600 μg dexamethasone (top panels) or from noninjected controls (lower panel). Gating areas for the CD4 versus CD8 dot plots were either CD3-high or CD3-low thymocytes.

(E) Three-color flow cytometric analysis of CD4 and CD8β.1+2 and CD8β.2 expression on thymocytes isolated from either +/+ (upper panel) or +Δ1Δ2 (lower panel) (AKR.1 × C57B/6-129) F1 mice. Numbers in the dot plot quadrants indicate the percentage of the corresponding thymocyte subpopulations (of total gated thymocytes).

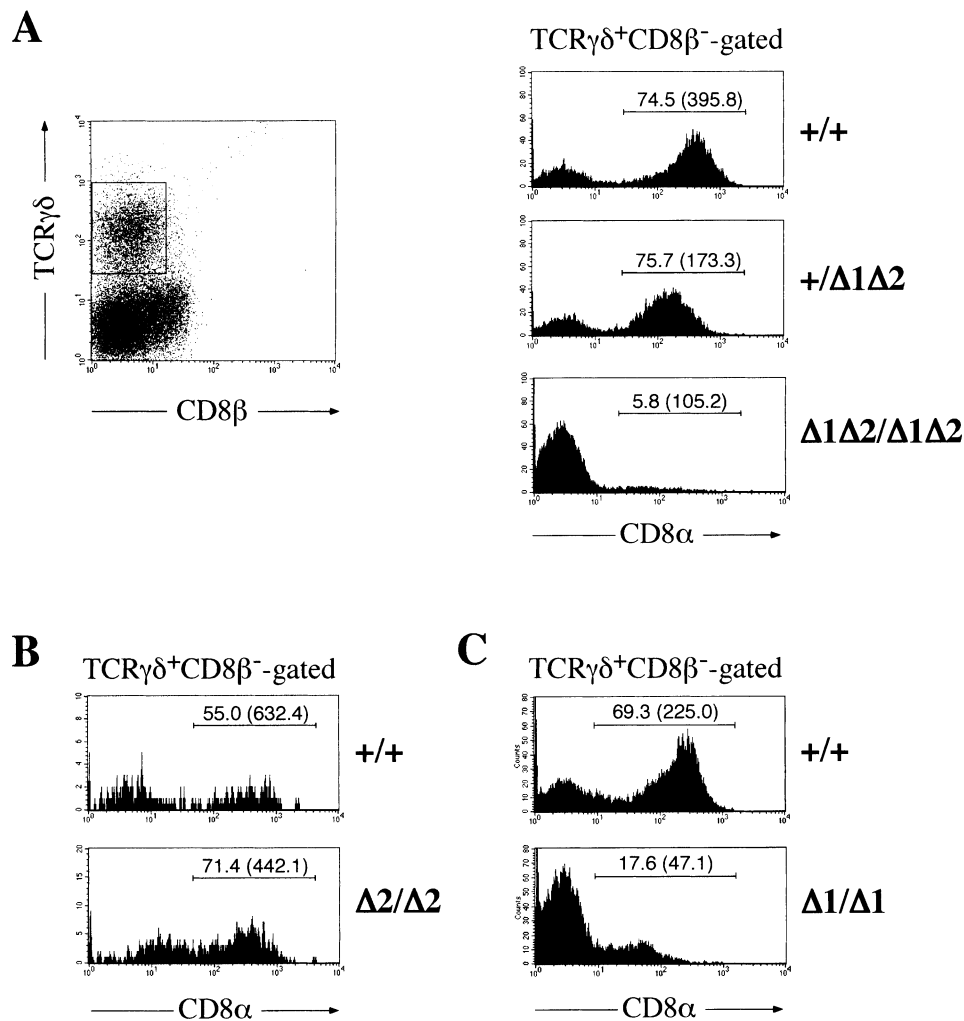


Figure 6. Enhancers E8_I (CIII-1,2) and E8_{II} (CIV-4,5) Regulate the Expression of CD8 α on TCR $\gamma\delta$ IEL

Flow cytometric analysis of intestinal intraepithelial lymphocytes isolated from the various enhancer knockout mice. Cells were analyzed for the expression of CD8 α , CD8 β , and TCR $\gamma\delta$.

(A) Histograms (right panel) show CD8 α expression on gated TCR $\gamma\delta^+$ (and CD8 β^-) IEL isolated from +/+, +/- Δ 1 Δ 2, and Δ 1 Δ 2/ Δ 1 Δ 2 littermates. The gate for TCR $\gamma\delta^+$ IEL is shown in the dot plot (left panel).

(B) Histograms show CD8 α expression on gated TCR $\gamma\delta^+$ (and CD8 β^-) IEL isolated from +/+ and Δ 2/ Δ 2 littermates.

(C) Histograms show CD8 α expression on gated TCR $\gamma\delta^+$ (and CD8 β^-) IEL isolated from +/+ and Δ 1/ Δ 1 littermates (as previously shown in Ellmeier et al., 1998). For (B) and (C), a representative gate used for TCR $\gamma\delta^+$ IEL is shown in the dot plot in (A). The numbers above the marked regions in the histograms indicate the percentage of CD8 α -positive cells within the gated populations, and the mean fluorescence of CD8 α expression levels is shown in brackets.

fewer DP thymocytes were present and fewer mature CD8 $^+$ T cells developed. These results reveal a novel function of enhancers that cannot be predicted by transgenic reporter analyses. They additionally suggest that there is partial redundancy of enhancers involved in initiation of CD8 gene expression in ISP or DP thymocytes. Even in the absence of both E8_I (CIII-1,2) and E8_{II} (CIV-4,5), a majority of thymocytes expressed CD8 in the DP compartment, suggesting that after initiation of expression, an epigenetic mechanism keeps the CD8 locus in an “on” configuration.

CD8 Expression in Thymocytes, Mature T Cells, and IEL

In contrast to the CD4 gene, which is regulated by developmental stage-specific activation of a silencer (Zou et

al., 2001), the CD8 locus appears to be under the complex control of a series of enhancer elements (Ellmeier et al., 1999). Based on the results from the transgenic reporter expression assays, one would predict that individual deletions of either enhancer E8_I (CIII-1,2) or E8_{II} (CIV-4,5) should lead (at least) to a reduction of CD8 expression levels in peripheral cytotoxic T cells, while the E8_{II} (CIV-4,5) deletion should also affect the expression of CD8 in the thymus. Conversely, CD8 α expression on IEL should be affected only by the deletion of E8_I (CIII-1,2) but not of E8_{II} (CIV-4,5). Furthermore, the combined deletion of both E8_I (CIII-1,2) and E8_{II} (CIV-4,5) should lead to a more dramatic reduction of CD8 levels than the individual deletions. However, the normal expression of CD8 in thymus-derived T cell lineages in E8_I (CIII-1,2)-deficient (Δ 1/ Δ 1) mice indicated already

that enhancer activities exist which can fully compensate for loss of $E8_I$ (CIII-1,2) (Ellmeier et al., 1998; Hostert et al., 1998). $E8_{II}$ (CIV-4,5) directs expression in DP and CD8 SP thymocytes as well as mature $CD8^+$ T cells (Ellmeier et al., 1998), and thus the finding of normal CD8 expression in its absence indicates compensatory enhancer activities not only in peripheral $CD8^+$ T cells but also in developing thymocytes. Since other enhancers such as the mature CD8 enhancer $E8_I$ (CIII-1,2) or the DP-specific enhancer $E8_{III}$ (CIV-3) are able to direct expression in similar T cell subsets as $E8_{II}$ (CIV-4,5) does (Ellmeier et al., 1999), it is possible that $E8_I$ (CIII-1,2) and $E8_{III}$ (CIV-3) can compensate for loss of $E8_{II}$ (CIV-4,5) in mature CD8 T cells and DP thymocytes, respectively. Based on the unexpected finding of largely normal CD8 expression in mature T cells of $\Delta 1\Delta 2/\Delta 1\Delta 2$ mice, however, it is likely that additional enhancers can function in these cells to direct expression of $CD8\alpha$ and $CD8\beta$. In addition, the observation of variegated expression of CD8 in $\Delta 1\Delta 2/\Delta 1\Delta 2$ DP thymocytes, which is not predicted by the transgenic results, suggests additional functions of the genomic enhancer fragments that cannot be compensated by the activity of other *cis* elements.

We previously showed that $E8_I$ (CIII-1,2) is able to direct expression in $CD8\alpha\alpha^+$ IEL in transgenic mice (Ellmeier et al., 1997). Targeted deletion confirmed that $E8_I$ (CIII-1,2) is the major enhancer that directs expression of $CD8\alpha$ in this lineage (Ellmeier et al., 1998; Hostert et al., 1998). A subset of $CD8\alpha\alpha$ -expressing $TCR\gamma\delta^+$ IEL in $\Delta 1/\Delta 1$ mice, however, still expressed $CD8\alpha\alpha$ homodimers (although at lower levels) on their surface (Ellmeier et al., 1998). One possible explanation was that other (unknown) enhancer elements are able to compensate for loss of $E8_I$ (CIII-1,2) in this particular $CD8\alpha\alpha^{low}TCR\gamma\delta^+$ IEL subset. Surprisingly, enhancer $E8_{II}$ (CIV-4,5), which did not show any activity in $CD8\alpha\alpha^+$ IEL in transgenic mice (Ellmeier et al., 1998), was found to compensate, at least partially, for loss of $E8_I$ (CIII-1,2). An almost complete absence of $CD8\alpha$ expression in $TCR\gamma\delta^+$ IEL and a further reduction of $CD8\alpha$ expression in $CD8\alpha\alpha^+TCR\alpha\beta^+$ IEL were observed in $\Delta 1\Delta 2/\Delta 1\Delta 2$ mice. $E8_I$ (CIII-1,2) and $E8_{II}$ (CIV-4,5) are therefore the *cis*-regulatory elements that direct expression of $CD8\alpha$ in $CD8\alpha\alpha$ homodimer-expressing IEL of the $TCR\gamma\delta$ lineage. In $CD8\alpha\alpha^+$ IEL of the $TCR\alpha\beta$ lineage, additional elements are able to direct low-level expression of $CD8\alpha$.

Thus, although transgenic analysis of reporter genes has suggested that enhancers may function in sequential stage-specific activation of the locus, the results presented here suggest a more complex mechanism. $E8_I$ (CIII-1,2) appears to contribute to establishment of CD8 expression in immature thymocytes even though it fails to direct expression of reporter genes in thymus; similarly, $E8_{II}$ (CIV-4,5) contributes to expression in IEL that is only revealed by analysis of mice with compound mutations.

How Do CD8 Enhancers Function during T Cell Development?

The finding of “CD8-negative” SP thymocytes and their developmental relationship to DP cells in $\Delta 1\Delta 2/\Delta 1\Delta 2$ mice may provide key insight into how the *CD8* genes are regulated in the thymus. Since these cells were present

even in the absence of $TCR\alpha$, alterations in CD8 expression must occur before the onset of positive selection. The appearance of “CD8-negative” SP cells points therefore to at least three potential mechanisms to explain how differentiating thymocytes may employ the various *CD8* enhancers during the developmental window between the pre-TCR stage (β selection) and positive selection.

An explanation for the appearance of the “CD8-negative” SP cells that we cannot exclude at the present time is that there are at least two distinct subsets of DP thymocytes. One subset would require $E8_I$ (CIII-1,2) and $E8_{II}$ (CIV-4,5), while the other(s) would utilize different *cis*-regulatory elements to express CD8 [such as $E8_{III}$ (CIV-3) and/or $E8_{IV}$ (CIV-1,2) or other unidentified elements]. This would be an unexpected finding, since it is generally assumed that DP thymocytes are a homogeneous population before the onset of positive selection, at least with respect to CD4 and CD8 expression and their potential to develop into the helper or cytotoxic lineages. A comparative analysis of the “CD8-negative SP” and DP thymocytes on a $TCR\alpha$ -deficient background, e.g., by means of expression profiling, may help to reveal whether subsets of DP thymocytes can be separated based on differential utilization of CD8 enhancers.

A second possibility is that the onset of CD8 expression is delayed in $\Delta 1\Delta 2/\Delta 1\Delta 2$ DN thymocytes, and therefore a large fraction of thymocytes initially expresses only CD4. During developmental progression, other CD8 enhancers such as $E8_{III}$ (CIV-3) may become activated and direct expression of CD8 in DP thymocytes. This “enhancer switching” model implies that developing thymocytes utilize different enhancers during their progression through the DP stage. Either $E8_I$ (CIII-1,2) or $E8_{II}$ (CIV-4,5) would be required at earlier stages of DP thymocyte development, while at later stages DP cells would utilize other *cis*-regulatory elements. This model predicts that “CD8-negative” SP cells are precursors of DP thymocytes and therefore appear before DP thymocytes develop. This is not observed in FTOC experiments, in which “CD8-negative” SP thymocytes and DP cells appeared simultaneously. Similar observations have also been made when a new wave of DP thymocyte development was monitored in $\Delta 1\Delta 2/\Delta 1\Delta 2$ mice after the depletion of DP cells by *in vivo* administration of dexamethasone (W.E., unpublished data). In addition, short-term BrdU labeling studies in mice showed that both “CD8-negative” SP cells and DP thymocytes incorporated BrdU to a similar extent. Thus, the enhancer switching model insufficiently explains the presence of “CD8-negative” SP thymocytes in $\Delta 1\Delta 2/\Delta 1\Delta 2$ thymocytes.

We favor an alternative explanation to describe the phenotype of $\Delta 1\Delta 2/\Delta 1\Delta 2$ mice, which is that $E8_I$ (CIII-1,2) and $E8_{II}$ (CIV-4,5) are involved in the initiation of CD8 expression in DN thymocytes. In their absence, thymocytes may only incompletely (i.e., only with a certain probability) initiate expression of CD8, and thus only a certain percentage of DP cells express CD8. This variegated expression of CD8 in the DP compartment is reminiscent of position effect variegation observed for transgenes (Festenstein and Kioussis, 2000). Thus, $E8_I$ (CIII-1,2) and $E8_{II}$ (CIV-4,5) may participate in the initiation of CD8 expression after β selection, e.g., by

mediating chromatin remodeling at the *CD8* locus due to the recruitment of chromatin opening activities. Once CD8 expression is established, thymocytes and mature CD8⁺ T cells would then maintain high-level stable expression of CD8 by a mechanism currently unknown. According to this "initiation/maintenance model," "CD8-negative" SP and DP thymocytes in $\Delta 1\Delta 2/\Delta 1\Delta 2$ mice should develop simultaneously and should also display a similar proliferative state. This is in agreement with observations made with FTOC experiments and BrdU labeling studies, respectively. Variegation of CD8 expression is observed as early as at the ISP stage in FTOC. Thus, thymocytes that do not express CD8 in these precursors may be those that proceed directly to the "CD8-negative" CD4 SP phenotype, which would indicate that an epigenetic decision occurs at this earlier stage.

Are Additional *cis* Elements Required for Initiating CD8 Expression?

The initiation/maintenance model implies that some of the CD8 *cis*-regulatory elements may not be required to direct but rather to facilitate transcription (e.g., by opening up the CD8 locus and thereby allowing transcription factors to bind to other *cis* elements). Similar observations that different *cis* elements mediate different aspects in the regulation of gene expression (such as opening of a gene locus versus high-level expression) have also been described for the β -*globin* locus (Bender et al., 2000; Engel and Tanimoto, 2000; Higgs, 1998). Since most thymocytes still express CD8 (along with CD4) in the absence of E8_i (CIII-1,2) and E8_{ii} (CIV-4,5), additional elements at the *CD8* locus must be involved in facilitating CD8 expression. A candidate region for such an element might be the DNase I hypersensitivity (DH) site cluster II (CII-1,2,3) located immediately upstream of the *CD8 α* gene. Cluster II cannot direct expression of a reporter gene by itself (Hostert et al., 1997a), but in combination with cluster III/E8_i (CIII-1,2) (see Figure 1A) it directs expression not only in mature CD8⁺ T cells but also in DP thymocytes (Hostert et al., 1998). Targeted deletion of cluster II leads to a similar phenotype as observed in $\Delta 1\Delta 2/\Delta 1\Delta 2$ mice (Garefalaki et al., 2002), indicating that this region may indeed function as another regulatory element that facilitates the expression of CD8 in DP thymocytes.

Maintenance of CD8 Expression

An important question that remains to be addressed is how the expression of CD8 is maintained in $\Delta 1\Delta 2/\Delta 1\Delta 2$ DP and CD8 SP thymocytes, and in mature CD8⁺ T cells. The expression of CD8 in $\Delta 1\Delta 2/\Delta 1\Delta 2$ DP thymocytes appears to remain stable even in the absence of positive selection, since $\Delta 1\Delta 2/\Delta 1\Delta 2$ DP TCR α null thymocytes reexpress both CD4 and CD8 after pronase treatment (W.E., unpublished data). One possibility is that after the establishment of an open CD8 locus, additional *cis* elements, such as E8_{iii} (CIV-3), E8_{iv} (CIV-1,2), or yet unknown enhancers, are sufficient to direct expression of CD8. Alternatively, but not mutually exclusive, chromatin remodeling events and subsequent establishment of CD8 expression in thymocytes may result in an epigenetic change that keeps the *CD8* locus active even in

the absence of a specific mature enhancer. This would not be unprecedented in the regulation of coreceptor gene expression, since it has been recently shown that the CD4 silencer is required for the establishment but not the maintenance of CD4 silencing (Zou et al., 2001). However, additional developmental stage-specific enhancer activities, and therefore (at least) partial redundancy, must exist. According to recent studies, DP thymocytes that have received signals for positive selection terminate CD8 expression, even if they differentiate into CD8⁺ T cells (Brugnera et al., 2000). Thus, thymocytes that develop into cytotoxic T cells utilize lineage- and developmental stage-specific regulatory elements to re-express CD8 ("coreceptor reversal"). One candidate element involved in the reexpression of CD8 is the mature CD8 enhancer E8_i (CIII-1,2), which in transgenic mice gets activated during positive selection. However, previous studies (Ellmeier et al., 1998; Hostert et al., 1998) and also this study indicate that in the absence of E8_i (CIII-1,2) mature T cells are still able to reexpress CD8. In addition, E8_i (CIII-1,2) gets activated at the CD3^{hi}HSA^{lo} stage (Ellmeier et al., 1997; Hostert et al., 1997b), thus, late during positive selection. Therefore, it is predicted that additional regulatory elements (or combined activities) activate CD8 in maturing CD8 lineage cells and may be operative even in the absence of E8_i (CIII-1,2). Since at the endogenous *CD8* locus, synergistic combinatorial interactions occur between different *cis*-regulatory sequences, further combined deletions of *cis* regions need to be performed to reveal the identity of potential immature and mature enhancer combinations.

Experimental Procedures

Generation of +/ $\Delta 2$ and +/N1 $\Delta 2$ ES cells

Genomic clones containing various fragments from the *CD8* locus were isolated from a 129 genomic library (Stratagene) and subcloned into pBluescript (pBS; Stratagene). The short (as a 0.9 kb BamHI/HindIII fragment) and the long (as a 6.0 kb BamHI/ClaI fragment) arms of the E8_{ii} (CIV-4,5) targeting construct were sequentially cloned into a pBS-based vector containing a polylinker with suitable cloning sites and the thymidine kinase gene driven by the herpes simplex virus enhancer/promoter elements (Thomas and Capecchi, 1990). The neomycin resistance gene expression cassette flanked by two loxP sites (Gu et al., 1993) was subsequently inserted (as a 1.4 kb XhoI/SalI fragment) between the two arms. All cloning steps were performed according to standard procedures. E14.1 ES cells (Kuhn et al., 1991) were transfected as previously described (Ellmeier et al., 1998), and proper targeting of ES cells (designated +/N2) was detected by Southern blotting. The deletion of the neomycin resistance gene was achieved by transient transfection of 20 μ g Cre recombinase into +/N2 ES cells. Two days after Cre transfection, ES cells were replated onto 10 cm dishes at a density of 300 colonies/plate. Individual clones were isolated, expanded, and G418-sensitive ES cells were analyzed by Southern blotting to confirm proper deletion of neomycin (designated +/ $\Delta 2$).

For the generation of +/N1 $\Delta 2$ ES cells, +/ $\Delta 2$ ES cells were transfected with the E8_i (CIII-1,2) targeting vector and screened as previously described (Ellmeier et al., 1998). After the isolation of single colonies, Southern blot analysis was performed to confirm proper targeting (Ellmeier et al., 1998) and also that both enhancers were targeted on the same chromosomal allele. The various ES cell clones (+/ $\Delta 2$ and +/N1 $\Delta 2$) were injected into E3.5 C57Bl/6 blastocysts and transferred into (B6/D2) F1 pseudo-pregnant females. Chimeric mice obtained were then backcrossed to C57Bl/6, and transmission of the targeted allele was confirmed by PCR and Southern blot analyses of tail DNA. The neomycin expression cassette at the E8_i (CIII-1,2) locus was deleted by crossing heterozygous +/N1 $\Delta 2$ mice with

transgenic mice expressing Cre recombinase under the control of the CMV promoter (White et al., 1997). The deletion of neomycin was confirmed by PCR analysis (PCR products 2 and 3 in Figure 1, Ellmeier et al., 1998) and by Southern blotting.

Flow Cytometric Analysis and Antibodies

Cell suspensions were prepared from thymus, lymph nodes, or spleen and stained with the appropriate antibodies. The following antibodies were used: PE-anti-mCD8 α (CT-CD8 α), FITC- or PE-anti-mCD8 β .1+2 (CT-CD8 β), FITC-anti-mCD69 (H1.2F3), TC-anti-mCD4 (CT-CD4), FITC-anti-mCD3 (Clone 500-A2), PE-anti-B220 (RA3-6B2), bio-anti-mTCR $\gamma\delta$ (GL3), TC-streptavidin and FITC-streptavidin from Caltag, FITC- or bio-anti-HSA (M1/69), FITC-anti-mCD8 β .2 (53-5.8) bio-anti-CD69 (H1.2F3), bio-anti-mTCR $\gamma\delta$ (GL3), PE-anti-CD5 (53-7.3), and bio-anti-mTCR $\alpha\beta$ (H57-597) from Pharmingen, and FITC-anti-BrdU from Becton Dickinson. Cells were analyzed using Becton Dickinson FACScan flow cytometer and Cell Quest software.

Fetal Thymic Organ Culture

Thymic lobes from embryos (E14.5) of various genotypes were isolated and cultured in a humidified box on 0.45 μ m nylon filters (Millipore) on top of gel foam (Upjohn) at 37°C in RPMI-10% FCS. At various time points during the culture period, single-cell suspensions were made from individual thymic lobes and stained with appropriate antibodies for FACS analysis.

Dexamethasone Injections

Mice were injected i.p. with 600 μ g Dexamethasone (Sigma) in 500 μ l PBS. Cell suspensions were made from the thymus at various and stained with appropriate antibodies for FACS analysis.

Isolation of Intestinal Intraepithelial Lymphocytes

IEL were isolated and purified by 37% Percoll centrifugation (at 1750 rpm for 30 min at room temperature) as described previously (Ellmeier et al., 1997). Cells were washed twice with staining buffer, incubated for 5 min with Fc-block (Pharmingen), and subsequently stained with antibodies.

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References

Basson, M.A., and Zamoyska, R. (2000). The CD4/CD8 lineage decision: integration of signalling pathways. *Immunol. Today* 21, 509–514.

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.

Bender, M.A., Bulger, M., Close, J., and Groudine, M. (2000). β -globin gene switching and DNase I sensitivity of the endogenous β -globin locus in mice do not require the locus control region. *Mol. Cell* 5, 387–393.

Berg, L.J., and Kang, J. (2001). Molecular determinants of TCR expression and selection. *Curr. Opin. Immunol.* 13, 232–241.

Brugnera, E., Bhandoola, A., Cibotti, R., Yu, Q., Guinter, T.I., Yamashita, Y., Sharow, S.O., and Singer, A. (2000). Coreceptor reversal in the thymus: signaled CD4⁺8⁺ thymocytes initially terminate CD8 transcription even when differentiating into CD8⁺ T cells. *Immunity* 13, 59–71.

Chan, S.H., Waltzinger, C., Baron, A., Benoist, C., and Mathis, D. (1994). Role of coreceptors in positive selection and lineage commitment. *EMBO J.* 13, 4482–4489.

Corbella, P., Moskophidis, D., Spanopoulou, E., Mamalaki, C., Tolaini, M., Itano, A., Lans, D., Baltimore, D., Robey, E., and Kioussis, D. (1994). Functional commitment to helper T cell lineage precedes positive selection and is independent of T cell receptor MHC specificity. *Immunity* 7, 269–276.

Davis, C.B., Killeen, N., Crooks, M.E., Raulet, D., and Littman, D.R. (1993). Evidence for a stochastic mechanism in the differentiation of mature subsets of T lymphocytes. *Cell* 73, 237–247.

Donda, A., Schulz, M., Burki, K., De Libero, G., and Uematsu, Y. (1996). Identification and characterization of a human CD4 silencer. *Eur. J. Immunol.* 26, 493–500.

Ellmeier, W., Sunshine, M.J., Losos, K., Hatam, F., and Littman, D.R. (1997). An enhancer that directs lineage-specific expression of CD8 in positively selected thymocytes and mature T cells. *Immunity* 7, 537–547.

Ellmeier, W., Sunshine, M.J., Losos, K., and Littman, D.R. (1998). Multiple developmental stage-specific enhancers regulate CD8 expression in developing thymocytes and in thymus-independent T cells. *Immunity* 9, 485–496.

Ellmeier, W., Sawada, S., and Littman, D.R. (1999). The regulation of CD4 and CD8 coreceptor gene expression during T cell development. *Annu. Rev. Immunol.* 17, 523–554.

Engel, J.D., and Tanimoto, K. (2000). Looping, linking, and chromatin activity: new insights into beta-globin locus regulation. *Cell* 100, 499–502.

Festenstein, R., and Kioussis, D. (2000). Locus control regions and epigenetic chromatin modifiers. *Curr. Opin. Genet. Dev.* 10, 199–203.

Garefalaki, A., Coles, M., Hirschberg, S., Mavria, G., Norton, T., Hostert, A., and Kioussis, D. (2002). Variegated expression of CD8 α resulting from in situ deletion of regulatory sequences. *Immunity* 16, this issue, 635–647.

Gorman, S.D., Sun, Y.H., Zamoyska, R., and Parnes, J.R. (1988). Molecular linkage of the Ly-3 and Ly-2 genes. Requirement of Ly-2 for Ly-3 surface expression. *J. Immunol.* 140, 3646–3653.

Gu, H., Zou, Y.R., and Rajewsky, K. (1993). Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. *Cell* 73, 1155–1164.

Higgs, D.R. (1998). Do LCRs open chromatin domains? *Cell* 95, 299–302.

Hostert, A., Tolaini, M., Festenstein, R., McNeill, L., Malissen, B., Williams, O., Zamoyska, R., and Kioussis, D. (1997a). A CD8 genomic fragment that directs subset-specific expression of CD8 in transgenic mice. *J. Immunol.* 158, 4270–4281.

Hostert, A., Tolaini, M., Roderick, K., Harker, N., Norton, T., and Kioussis, D. (1997b). A region in the CD8 gene locus that directs expression to the mature CD8 T cell subset in transgenic mice. *Immunity* 7, 525–536.

Hostert, A., Garefalaki, A., Mavria, G., Tolaini, M., Roderick, K., Norton, T., Mee, P.J., Tybulewicz, V.L.J., Coles, M., and Kioussis, D. (1998). Hierarchical interactions of control elements determine CD8 α gene expression in subsets of thymocytes and peripheral T cells. *Immunity* 9, 497–508.

Jarry, A., Cerf-Bensussan, N., Brousse, N., Selz, F., and Guy-Grand, D. (1990). Subsets of CD3⁺ (T cell receptor $\alpha\beta$ or $\gamma\delta$) and CD3⁻ lymphocytes isolated from normal human gut epithelium display phenotypical features different from their counterparts in peripheral blood. *Eur. J. Immunol.* 20, 1097–1103.

Kuhn, R., Rajewsky, K., and Muller, W. (1991). Generation and analysis of interleukin-4 deficient mice. *Science* 254, 707–710.

Lefrancois, L. (1991). Phenotypic complexity of intraepithelial lymphocytes of the small intestine. *J. Immunol.* 147, 1746–1751.

Leung, R.K., Thomson, K., Gallimore, A., Jones, E., Van Den Broek, M., Sierro, S., Alsheikhly, A.R., McMichael, A., and Rahemtulla, A. (2001). Deletion of the CD4 silencer element supports a stochastic

- mechanism of thymocyte lineage commitment. *Nat. Immunol.* **2**, 1167–1173.
- Mombaerts, P., Clarke, A.R., Rudnicki, M.A., Iacomini, J., Itoharu, S., Lafaille, J.J., Wang, L., Ichikawa, Y., Jaenisch, R., Hooper, M.L., et al. (1992). Mutations in T-cell antigen receptor genes alpha and beta block thymocyte development at different stages. *Nature* **360**, 225–231.
- Philpott, K.L., Viney, J.L., Kay, G., Rastan, S., Gardiner, E.M., Chae, S., Hayday, A.C., and Owen, M.J. (1992). Lymphoid development in mice congenitally lacking T cell receptor alpha beta-expressing cells. *Science* **256**, 1448–1452.
- Robey, E., Itano, A., Fanslow, W.C., and Fowlkes, B.J. (1994). Constitutive CD8 expression allows inefficient maturation of CD4⁺ helper T cells in class II major histocompatibility complex mutant mice. *J. Exp. Med.* **179**, 1997–2004.
- Rothenberg, E.V. (2000). Stepwise specification of lymphocyte developmental lineages. *Curr. Opin. Genet. Dev.* **10**, 370–379.
- Sawada, S., and Littman, D.R. (1991). Identification and characterization of a T-cell-specific enhancer adjacent to the murine CD4 gene. *Mol. Cell. Biol.* **11**, 5506–5515.
- Sawada, S., Scarborough, J.D., Killeen, N., and Littman, D.R. (1994). A lineage-specific transcriptional silencer regulates CD4 gene expression during T lymphocyte development. *Cell* **77**, 917–929.
- Siu, G., Wurster, A.L., Duncan, D.D., Soliman, T.M., and Hedrick, S.M. (1994). A transcriptional silencer controls the developmental expression of the CD4 gene. *EMBO J.* **13**, 3570–3579.
- Sleckman, B.P., Gorman, J.R., and Alt, F.W. (1996). Accessibility control of antigen-receptor variable-region gene assembly: role of cis-acting elements. *Annu. Rev. Immunol.* **14**, 459–481.
- Swat, W., Dessing, M., von Boehmer, H., and Kiseiow, P. (1993). CD69 expression during selection and maturation of CD4⁺8⁺ thymocytes. *Eur. J. Immunol.* **23**, 739–746.
- Thomas, K.R., and Capecchi, M.R. (1990). Targeted disruption of the murine int-1 proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. *Nature* **346**, 847–850.
- van Meerwijk, J.P., and Germain, R.N. (1993). Development of mature CD8⁺ thymocytes: selection rather than instruction? *Science* **261**, 911–915.
- von Boehmer, H. (2000). T-cell lineage fate: instructed by receptor signals? *Curr. Biol.* **10**, R642–R645.
- White, J.K., Auerbach, W., Duyao, M.P., Vonsattel, J.P., Gusella, J.F., Joyner, A.L., and MacDonald, M.E. (1997). Huntingtin is required for neurogenesis and is not impaired by the Huntington's disease CAG expansion. *Nat. Genet.* **17**, 404–410.
- 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.
- Zhang, X.L., Seong, R., Piracha, R., Larjani, M., Heeney, M., Parnes, J.R., and Chamberlain, J.W. (1998). Distinct stage-specific cis-active transcriptional mechanisms control expression of T cell coreceptor CD8 α at double- and single- positive stages of thymic development. *J. Immunol.* **161**, 2254–2266.
- Zou, Y.R., Sunshine, M.J., Taniuchi, I., Hatam, F., Killeen, N., and Littman, D.R. (2001). Epigenetic silencing of CD4 in T cells committed to the cytotoxic lineage. *Nat. Genet.* **29**, 332–336.