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*J Immunol* 2014; 192:4620-4627; Prepublished online 11 April 2014;
doi: 10.4049/jimmunol.1302374
http://www.jimmunol.org/content/192/10/4620

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**Supplementary Material**

http://www.jimmunol.org/content/suppl/2014/04/11/jimmunol.1302374.DCSupplemental

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A Silencer-Proximal Intrinsic Region Is Required for Sustained CD4 Expression in Postselection Thymocytes

David M. Henson,1 Chun Chou,1 Nagisa Sakurai, and Takeshi Egawa

It has been proposed that differential kinetics of CD4/CD8 coreceptors regulate fate choice of selected thymocytes. Sustained signals by interaction between MHC class II and TCR/CD4 is required for commitment to the CD4 helper lineage. Although prematurely terminated MHC–TCR/CD4 interaction in transgenic mouse models results in lineage redirection, it is unclear whether CD4 expression is actively maintained by endogenous cis-elements to facilitate prolonged signaling under physiological conditions. In this article, we show that sustained CD4 expression in postselection thymocytes requires an intronic sequence containing an uncharacterized DNase I hypersensitivity (DHS) site located 3′ to the silencer. Despite normal CD4 expression before selection, thymocytes lacking a 1.5-kb sequence in intron 1 including the 0.4-kb silencer and the DHS, but not the 0.4-kb silencer alone, failed to maintain CD4 expression upon positive selection and are redirected to the CD8 lineage after MHC class II–restricted selection. Furthermore, CpG dinucleotides adjacent to the DHS are hypermethylated in CD8+ T cells. These results indicate that the 1.5-kb cis-element is required in postselection thymocytes for helper lineage commitment, presumably mediating the maintenance of CD4 expression, and suggest that inactivation of the cis-element by DNA methylation may contribute to epigenetic Cd4 silencing. The Journal of Immunology, 2014, 192: 4620–4627.

CD4 and CD8 coreceptor expression defines subsets of mature T cells with distinct functions. Both CD4+ Th and CD8+ CTL are derived from a common precursor pool of CD4+ CD8+ (double-positive [DP]) thymocytes. As a consequence of random TCR-α chain rearrangements and interaction with MHC–peptide complexes presented on thymic epithelial cells, thymocytes expressing MHC class II (MHCIΙ)– or MHC class I (MHCI)–restricted TCR with appropriate avidity differentiate into the Th or CTL lineage, respectively (1–3). However, it is not understood how positive selection signals regulate fates of selected thymocytes.

It has been proposed that distinct expression kinetics of CD4 and CD8 coreceptors during positive selection determine the Th versus CTL lineage choice (2). Among several models that have been proposed and experimentally tested, the kinetic signaling model by Singer and colleague (4) has been supported by several lines of experimental evidence using mouse genetics. Cd8a/Cd8b1 genes, which encode two subunits of the CD8 coreceptor, are regulated by multiple stage-specific enhancers (5). During positive selection, CD8 coreceptor is transiently downregulated because of reduced transcription of Cd8a/Cd8b1 regardless of TCR-MHC specificity, whereas CD4 expression remains constant or is promptly restored after transient downregulation at the DPbright stage. These distinct expression kinetics of the two coreceptors cause differential duration of positive selection signals. MHCIΙ-restricted thymocytes receive prolonged signal because of continued interaction between TCR/CD4 and MHCIΙ, whereas MHCI-restricted thymocytes lose signals as surface CD8 is downregulated. This model was further tested in transgenic mice or knock-in models, in which Cd4 was only transiently expressed under the control of Cd8a/Cd8b1 regulatory elements (6, 7). These experiments demonstrated that sustained CD4 expression is required for induction of ThPOK and commitment to the Th lineage after MHCIΙ-restricted selection. Transient signaling through CD4/TCR interacting with MHCIΙ–peptide complexes results in redirection to the CTL lineage. However, these experiments were performed exclusively in genetically modified mouse models to drive Cd4 expression under the control of heterologous regulatory elements, and it therefore remains unknown whether Cd4 expression is actively maintained through activation of endogenous regulatory elements to permit prolonged signals.

Cis-regulatory elements that are required or sufficient for Cd4 expression have been identified and validated by genetic experiments (8, 9). Cd4 expression in developing thymocytes and T cells are predominantly regulated by a 0.35-kb Cd4 proximal enhancer (Ep4) located 12 kb 5′ to the promoter and a 0.4-kb silencer in the first intron. Deletion of the silencer results in ectopic expression of CD4 in DN thymocytes and MHCI-restricted CD8+ T cells (10, 11). Transgenic constructs under the control of the Cd4 promoter (P4) and Ep4 are expressed in all thymocyte subsets and mature T cells, indicating that Ep4 is constitutively active during T cell development and that the silencer activity defines stage- and lineage-specific Cd4 expression (10, 11). Targeted deletion of Ep4 abolishes Cd4 expression in DP thymocytes (8). Despite lack of CD4 expression in DP thymocytes, MHCI-restricted positive selection does occur in Ep4-deficient mice (8). These MHCI-restricted thymocytes turn on CD4, albeit at a lower level and frequency, and become ThPOK-expressing mature...
T cells, suggesting that Cd4 is driven by an unidentified enhancer, which becomes active after positive selection. This possibility is also supported by a previous study showing that an Ep4-P4 transgenic reporter is silenced in activated T cells without a change in endogenous CD4 expression (12).

In this study, we analyzed epigenetic marks in thymocytes and mature T cells, and examined the requirement of an intronic sequence containing a peak of DNaseI hypersensitivity (DHS) site located immediately 3′ to the 0.4-kb silencer and 3 kb downstream of the Cd4 promoter (DHS+3). Although deletion of the 0.4-kb silencer alone showed little impact on T cell lineage commitment, mice lacking a 1.5-kb intronic sequence encompassing both the silencer and DHS+3 failed to sustain CD4 expression in positively selected thymocytes, which resulted in lineage redirection of MHCII-restricted thymocytes to the CD8+ T cell lineage. Our results suggest that the Cd4 locus uses distinct cis-elements between preselection and postselection thymocytes, and that the sustained CD4 expression in postselection thymocytes, which requires the 1.5-kb sequence, or possibly the 1.1-kb sequence 3′ to the silencer, is essential for the lineage commitment to the helper lineage.

**Materials and Methods**

**Mice**

Cd4am2Lin (Cd4[0.4kb]−Δ0.4kb) and Cd4m1Yzo (Cd4[1.5kb]) alleles were described previously (11, 13). These alleles were backcrossed 12 generations to the C57BL6 (B6) background before intercrossing or crossing to Cre transgenic lines. Cd4am2Lin was bred to Cd4-cre (Tacicon) (14) to delete the 1.5-kb genomic fragment encompassing the silencer and DHS+3 (Δ1.5kb), or Vav1-cre (Jackson Laboratory) (15) for data shown in Supplemental Fig. 3. Mice homozygous for the Cd4am2Lin or Cd4m1Yzo were used for all experiments. C57BL6 mice (National Cancer Institutes, Frederic, MD) were used as wild-type control. Bone marrow chimeras were generated by i.v. transfer of bone marrow cells (5 × 106 cells) into lethally irradiated B6m−/− mice (16) purchased from Jackson Laboratory. The recipient mice were treated with trimethoprim and sulfamethoxazole for 4 wk and analyzed 12 wk after transplantation. All mice were housed in a specific pathogen-free animal facility at Washington University School of Medicine, and experiments were performed according to a protocol approved by the Animal Studies Committee at Washington University in St. Louis.

**Epigenetic analysis**

Data from deep sequencing analyses were previously published (17, 18) or have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus by the Encyclopedia of DNA Elements (ENCODE) Project. Data from the ENCODE Project were used in accordance with ENCODE Consortia Data Release, Data Use, and Publication Policies. FASTQ files or BigWig files were downloaded from Gene Expression Omnibus. FASTQ files were mapped to a mouse genome assembly mm9 using bowtie2 (19) and converted to TDF files. BigWig and TDF files were visualized in IGV browser (version 2.3; Broad Institute).

**Chromatin immunoprecipitation**

Native chromatin immunoprecipitation (ChIP) was performed by following a previously published protocol (20). In brief, native chromatin prepared from 5–10 × 10^6 cells was digested with micrococcal nuclease (Worthington) and subjected to immunoprecipitation usingAbs against acetylated histone H3 lysine 27 (H3K27ac; ab4729; Abcam) or dimethylated histone H3 lysine 4 (H3K4me2; ab7760; Abcam) coupled to Dynabeads protein G (Life Technologies). Precipitated DNA was purified, and enrichment of the Cd4 regulatory regions was quantitated by real-time PCR with the following primers using a Roche LC480II and Luminaris Color SYBR green PCR reagent (Thermo Fisher): Cd4Sii-F: 5′-TACGAAAGCTCAGAAAGGGAAG-3′; Cd4Sii-R: 5′-TGTGCTGCGATGCGGCTT-3′; Enhancer (Eb)-F: 5′-GGTTGAACGATCAGGATAATATGAGGAAAG-3′; EbsF3-5′-AAAGGCAAGAGGCGGATAGAAGGGAAGAG-3′; DHS+-3.5′-AAGGAGGAAGAGCCCAATAGAG-3′; DHS+3-R: 5′-TGTTTCATCCGGTTGATAGA-3′.

**Luciferase reporter assay**

The Cd4 intronic fragments were PCR amplified and cloned into KpnI and XhoI sites of a pGL3-Promoter plasmid expressing the firefly luciferase under the control of the SV40 promoter (Promega). Jurkat cells were cotransfected with each SV40-firefly luciferase plasmid and a CMV-RL plasmid (Promega) using a TransIT-Jurkat transfection reagent (Mirus Bio) according to the manufacturer’s instruction. Luciferase activities were measured 16 h after transfection.

**Statistical analysis**

Statistical differences were tested using GraphPad Prism 6.0 by two-tailed unpaired Student’s t test for two group comparisons or one-way ANOVA with the Tukey post hoc analysis for comparison between more than three groups. The p values <0.05 were considered significant: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

**Results**

The 1.5-kb intronic sequence containing the silencer and DHS+3 regulates CD4 expression in postselection thymocytes

To locate a potential regulatory element, we analyzed publicly available deep sequencing data for DHS and ChIP against permissive histone modifications in DP thymocytes and mature T cells (Fig. 1A). In total thymocytes, there were multiple DHS peaks in a genomic region encompassing the Cd4 locus, including two strong peaks corresponding to Ep4 and the promoter region for Lag3. A slightly weaker peak was found in the first intron of Cd4 immediately 3′ to the 0.4-kb silencer region (DHS+3). In mature T cells, although the Lag3 promoter peak remained high, the peak in intron 1 became dominant compared with the peak at Ep4, suggesting that the Cd4 locus is regulated by distinct elements between thymocytes and mature CD4+ T cells. Consistent with active chromatin states in ChIPseq data, both Cd4 silencer and DHS+3 were enriched for the H3K4me2 and H3K27ac marks in thymocytes and mature CD4+ T cells, but devoid of these marks in CD8+ T cells (Fig. 1B). Sequence analysis between mouse and human genomes using the ECR browser (http://ecrbrowser.dcode.org/) showed that DHS+3 is highly conserved between mouse and human to a similar degree to the 0.4-kb silencer and contained a cluster of predicted binding sites for the HNF family of nuclear receptors, TCF1–LEF1 complexes, STAT3, SRF1, c-REL, RUNX protein, WT1, and CACD factors (Supplemental Fig. 1). These results suggest a potential regulatory role of DHS+3 in regulation of Cd4 gene expression in developing thymocytes and T cells.
To determine whether the intronic region in the *Cd4* locus containing DHS+3 has the regulatory role in *Cd4* gene regulation in vivo, we examined CD4 expression and T cell development in mice lacking a 1.5-kb fragment (*D1.5kb, Cd4*tm1Yzo crossed to *Cd4*-cre) encompassing the 0.4-kb silencer and an additional 1.1-kb region containing DHS+3 and mice lacking only the 0.4-kb silencer (*D0.4kb; Fig. 2A). We did not observe differences in total thymus or spleen cellularity between age-matched wild-type B6, *D0.4kb*, and *D1.5kb* mice (Supplemental Fig. 2A). CD4 expression in preselection CD69+ TCRblo/2 CD24hi DP thymocytes, CD69+ CD24hi CD8+ postselection intermediate, and TCRbhi CD24lo/2 CD8+ mature thymocytes was grossly comparable between B6 and *D0.4kb* mice (Fig. 2B and Supplemental Fig. 2B). These results indicate that the 0.4-kb silencer is dispensable for normal CD4 expression in developing thymocytes in vivo. In contrast, despite normal expression in preselection thymocytes, the CD4 level was reduced in postselection intermediate and mature thymocytes in *D1.5kb* mice (Fig. 2B and Supplemental Fig. 2B). Besides reduced CD4 expression, the absolute number of TCRbhi CD24lo/2 CD4SP cells was reduced by 2-fold, and TCRbhi CD24lo/2 CD8+ mature thymocytes increased by 3-fold compared with B6 or *D0.4kb* mice (Fig. 2C). A similar decrease in CD4+ CD8lo T cells and increase in CD8+ T cells were observed in the spleen of *D1.5kb* mice (Fig. 2C). Total numbers of TCRbhi CD24lo/2 thymocytes were also increased by 1.5-fold in *D1.5 kb* mice compared with B6 or *D0.4kb* mice (Supplemental...
Fig. 2A). We observed nearly identical phenotypes when the 1.5-kb sequence was deleted with the Vav1-cre deleter (Supplemental Fig. 3), indicating that these phenotypes were not caused by the presence of multiple copies of Cd4 regulatory elements in the Cd4-cre transgenic mice. Unlike CD4+ T cells in ThPOK/Zbtb7b hypomorphic mutant (ThPOK FN) mice (22), the remaining CD4+ T cells in D1.5kb mice expressed ThPOK protein at a comparable level to B6 CD4+ T cells and did not express Runx3 (Supplemental Fig. 2C). They also expressed IFN-γ and IL-2 at comparable frequencies to control D0.4kb CD4+ CD8- or CD8+ T cells under nonpolarizing conditions (Supplemental Fig. 2D). These results indicate that the 1.5-kb intronic sequence containing the silencer and DHS+3 is required for sustained CD4 expression in postselection thymocytes and mature T cells, and that diminished CD4 expression after positive selection leads to reduced Th lineage differentiation.

To determine whether the intronic sequence deleted in Δ1.5kb mice is sufficient to mediate an enhancer activity, we inserted the 1.5- or 1.1-kb genomic sequence to an SV40 promoter–driven luciferase expression plasmid and transfected into Jurkat cells. Neither the 1.5-kb nor the 1.1-kb sequence showed an enhancement in luciferase expression (Fig. 2D), suggesting that the 1.5-kb sequence by itself is insufficient to provide an enhancer activity, and that other unknown sequences in the endogenous Cd4 locus cooperate with the deleted region to drive gene expression.

Deletion of the 1.5-kb sequence containing DHS+3 results in lineage redirection Reduced numbers of CD4SP thymocytes and CD4+ CD8- T cells, and increased numbers of CD8+ mature thymocytes and splenic CD8+ T cells in D1.5kb mice suggest lineage redirection of MHCII-restricted thymocytes as observed in ThPOK-deficient mice (22–25) or transgenic mice, in which Cd4 is expressed under the control of Cd8a/Cd8b1 regulatory elements (6, 7). To determine whether the 1.5-kb genomic region containing the silencer and DHS+3 is required for commitment to the CD4+ Th lineage, we generated bone marrow chimeras using B2m2/2 mice as recipients. In these chimeras, thymocytes are selected predominantly by MHCII. In chimeras receiving D0.4kb bone marrow cells (in ThPOKGFP background), the majority of mature thymocytes and splenic T cells were CD4+ CD8- (Fig. 3A and 3B). These CD4SP cells uniformly expressed the ThPOK-GFP
condition after 4 d of culture are shown with means and SD. Statistical differences were assessed by two-way ANOVA and the Tukey test. n = 4. (B) CD4 and GFP expression in Cre-expressing retrovirus (Cre-RV)–transduced B6 and Cd4(1.5k)F/F CD4+ T cells on day 4 after transduction and a histogram overlay of CFSE-labeled CD4+ CD8+ T cells under nondividing/slowly dividing (IL-7) and rapidly dividing (anti-CD3 and anti-CD28) conditions. Percentages of CD8+ mature thymocytes and T cells development but dispensable for maintenance of CD4 expression in activated T cells or from a constitutive requirement for the 1.5-kb sequence in maintenance of CD4 expression in activated T cells or from a defective establishment of stable CD4 expression during development in the absence of the 1.5-kb sequence. To test these possibilities, we used a retrovirus expressing Cre recombinase to delete the 1.5-kb sequence in mature CD4+ T cells and tracked CD4 expression in dividing cells. CD4 expression was comparable between control B6 CD4+ T cells and Cd4(1.5k)F/F CD4+ T cells after Cre-mediated deletion of the 1.5-kb sequence in activated CD4+ T cells (Fig. 4B and 4C), indicating that the 1.5-kb sequence is dispensable for the maintenance of CD4 expression in normally developed CD4+ T cells. These results also suggest that stable CD4 expression is established in Ep4 and the 1.5-kb sequence-dependent manners during thymocyte differentiation.
Hypermethylation of Cpg dinucleotides adjacent to DHS+3 in CD8\(^+\) T cells

During normal T cell development, CD8\(^+\) T cells epigenetically shut off Cd4 in a silencer-dependent manner (11, 13). Although epigenetic silencing is linked to DNA methylation (26), a previous study showed that there is no difference in Cpg methylation in the Cd4 promoter region between CD4\(^+\) and CD8\(^+\) T cells (13). We hypothesized that the silencer or DHS+3 could be a target of CD8\(^+\) T cell–specific DNA methylation, which may contribute to epigenetic Cd4 silencing. To test this hypothesis, we mapped CpG methylation in the 1.5-kb region containing the 0.4-kb silencer and DHS+3 by bisulfitite sequencing. Although the silencer was hypomethylated in both CD4\(^+\) and CD8\(^+\) T cells, a few CpG dinucleotides located immediately 3' to the DHS+3 peak were hypermethylated in CD8\(^+\) T cells (Fig. 5A). In contrast, CpG methylation at the same dinucleotides was substantially reduced in CD4\(^+\) T cells. To further test whether maintenance of Cd4 silencing requires DNA methylation, we treated CD8\(^+\) T cells from B6 mice with the DNA methyltransferase inhibitor, 5-AZA, and examined CD4 expression. We observed CD4 upregulation in CD8\(^+\) T cells treated with 5-AZA, even though the level of CD4 expression was substantially lower than control CD4\(^+\) T cells (Fig. 5B). These results suggest that DNA methylation may be required for maintenance of Cd4 silencing, and that DHS+3 could be a target of CD8\(^+\) T cell–specific DNA methylation.

Discussion

In this study, we characterized the requirement for the silencer and its neighboring DHS in the Cd4 gene regulation. Our data showed that a 1.5-kb intronic region containing DHS+3 and the previously established 0.4-kb silencer is required for sustained CD4 expression in postselection thymocytes and commitment of MHCII-restricted thymocytes to the helper lineage. Despite normal expression in preselection DP thymocytes, CD4 expression was diminished in CD69\(^+\) CD24\(^+\) postselection thymocytes in the absence of the 1.5-kb region, and a large proportion of MHCII-restricted thymocytes was redirected to the CD8 lineage. Because we did not observe CD4 downregulation in mice lacking the 0.4-kb silencer alone, sustained CD4 expression in postselection thymocytes is possibly mediated by the 1.1-kb sequence located immediate 3' to the 0.4-kb silencer. The 1.1-kb genomic region contains a DHS peak mapped to an evolutionarily conserved sequence and is marked by H3K27ac and H3K4me2, histone modifications known to be enriched in active enhancer regions. Although we could not detect an enhancer activity mediated by the 1.5- or 1.1-kb sequence in a luciferase reporter assay in vitro, our data suggest that the 1.5-kb intronic sequence is required, although not sufficient, for enhancer activity essential for sustained CD4 expression. This region itself may not function as a classical enhancer, which serves as a dock for transcriptional activator or coactivator binding. It may rather be necessary to establish a permissive chromatin conformation via functioning as a flexible hinge to place two or more distally located elements in a close proximity. Although we speculate that reduced CD4 expression in Δ1.5kb mice causes lineage redirection, we do not rule out the possibility that it acts in trans on other loci encoding molecules involved in helper versus cytotoxic lineage decisions. A previous study suggests that the 0.4-kb silencer and Runx proteins that bind to the silencer mediate association of Cd4 and Cd8b1 loci in thymocytes (27). Although a deletion of only the 0.4-kb silencer did not alter CD8 expression or cause marked lineage redirection, intrachromosomal or interchromosomal associations mediated by the 1.5-kb sequence, which contains the silencer element, may play a role in the helper lineage commitment.

In the kinetic signaling model, sustained positive selection signals instruct cells to differentiate into the CD4\(^+\) Th lineage, whereas an artificial interruption of MHCII-restricted positive selection signals by transgenic expression of CD4 results in lineage redirection (2, 6, 7). In cells lacking the 1.5-kb sequence, MHCII-restricted positive selection signals may be prematurely interrupted because of CD4 downregulation after selection is initiated, thus leading to insufficient ThPOK induction and the development of MHCII-restricted CD8\(^+\) cells. Our results suggest that the Cd4 locus may undergo enhancer switching in part similar to Cd8al/Cd8b1 loci, and that CD4 expression may actively be maintained by a postselection stage-specific enhancer that requires the 1.5-kb sequence. Although transcription factor requirements for CD4 versus CD8 lineage decision have been explained in large part by Gata3, ThPOK, and Runx3/CBFβ (3, 28–30), there may be additional factors that also play key roles in the cell fate decision process presumably through regulation of sustained Cd4 expression. The putative postselection enhancer appears likely to account for Ep4-independent CD4 expression after MHCII-restricted selection (8). Because CD4 expression was not completely inactivated in Δ1.5kb cells, the postselection enhancer activity may not be completely lost by the deletion, or other enhancers, including Ep4, may be at least partially redundant in postselection thymocytes. Interestingly, although CD4\(^+\) T cells from Cd4 (Ep4\(^{-}\)) or Δ1.5kb mice failed to establish stable CD4 expression, the requirements for the 1.5-kb sequence and Ep4 are transient, and they are not required for maintenance of CD4 expression. These findings suggest that both Ep4 and the silencer/DHS+3-containing cis-element have stage-specific functions during thymocyte development.
In addition to lineage redirection of MHCII-selected thymocytes to the CTL lineage, we also observed an ~1.5-fold increase in the number of total mature thymocytes with a relatively mild reduction of CD4SP thymocytes in Δ1.5kb mice compared with WT B6 or Δ0.4kb mice. A recent study using Nur-77 GFP reporter and Bim+/− mice demonstrated that more than half of MHCII-selected thymocytes that differentiate to CD4SP cells are negatively selected during normal thymocyte development (31). In Bim+/− mice, which are defective in negative selection, both CD4SP and CD8SP thymocytes substantially increase with an enrichment of Nur77th cells, which are normally eliminated by negative selection. Therefore, the increased number of mature thymocytes may reflect reduced negative selection resulting from CD4 downregulation in postselection thymocytes in Δ1.5kb mice, but not in Δ0.4kb or B6 mice.

Previously, Leung et al. (32) generated a mouse strain in which a larger proportion of Cd4 intron 1 was deleted. Because the 0.4-kb silencer was also deleted in this strain, CD8 T cells expressed CD4 similarly to the two lines of mice we used in this study. In addition to loss of Cd4 silencing in CD8 T cells, the study reported phenotypes similar to those we observed in Δ1.5 kb mice, including diminished CD4 expression, reduced CD4+ T cells, and increased CD8+ T cells caused by redirection of MHCII-restricted T cells to the CD8 lineage. The article was concluded in favor of the stochastic model of CD4 versus CD8 lineage decisions based on the authors’ interpretation that MHCII-restricted thymocytes that would have chosen to express CD8 and terminate CD4 were “rescued,” resulting in the development of MHCII-restricted CD8+ T cells. However, we argue against their interpretation because deletion of the 0.4-kb silencer alone, which allows both MHCII-restricted cells and MHCII-restricted cells to express high levels of CD4, resulted in the development of substantially reduced CD8+ T cells compared with mice with the 1.5-kb deletion. Based on these data, we concluded that the development of MHCII-restricted CD8+ T cells in our Δ1.5kb mice resulted primarily from reduced CD4 expression after positive selection, which compromised "prolonged" positive selection signals required for CD4 lineage commitment, rather than a rescue of "CD8-fated" MHCII-restricted thymocytes from cell death.

CpG dinucleotides adjacent to DHS+3 were hypermethylated in CD8+ T cells. This result could be interpreted in two different ways. In one possibility, inactivation of the putative postselection enhancer in CD8+ T cells may be important for epigenetic silencing. This is consistent with our observation that CD8+ T cells treated with 5-AZA derepressed CD4, although we are unable to rule out the possibility that 5-AZA treatment induced global gene expression changes that might indirectly cause CD4 derepression. The CD4 derepression occurred in the presence of the silencer, which may restrict CD4 derepression to only a modest extent. Synergistic effects by the silencer-dependent repression and inactivation of DHS+3 by DNA methylation may completely shut off the locus activity in CD8+ T cells. An alternative but not necessarily mutually exclusive possibility is that DHS+3 is demethylated specifically in CD4+ T cells, which allows full activation of the Cd4 locus activity. Future analysis examining de novo DNA methyltransferase or Tet family demethylase protein recruitment to the Cd4 locus may address this possibility.

In summary, our study provides genetic evidence that the 1.5-kb intronic sequence containing the silencer and DHS+3 serves as an essential cis-element for sustained CD4 expression in postselection thymocytes and lineage commitment to the CD4 Th lineage.

Acknowledgments

We thank Dan R. Littman (New York University School of Medicine, New York, NY), Yong-Rui Zou (Feinstein Institute for Medical Research, Manhasset, NY), Ichiro Taniuchi (RIKEN, Yokohama, Japan), and William E. Paul (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) for materials; Sunnie Hsiung for technical assistance; and Eugene Oltz and Chi-Yong Hsieh for critical reading of the manuscript.

Disclosures

The authors have no financial conflicts of interest.

References


Sequence conservation analysis of the 1.5 kb sequence encompassing the Cd4 silencer and DHS+3 between human and mouse genomes is shown as histogram and an alignment at the nucleotide level. Regions conserved >60% are shown in orange and repetitive sequences are shown in green as default in the ECR browser (http://ecrbrowser.dcode.org/). Sequence alignment at the nucleotide level is shown in the lower panel. The 0.4 kb silencer and the peak of DHS+3 shown in Fig. 1 are marked in purple and blue, respectively.
Supplementary Fig. 2. Analysis of T cell development in \( Cd4(0.4k)^{-/-} \) and \( Cd4(1.5k)^{F/F};Cd4-cre \) mice. (A) Statistical analysis of numbers of total thymocytes, total splenocytes, and TCR\( \beta^{hi} \) CD24\( ^{lo/-} \) mature thymocytes in B6, \( Cd4(0.4k)^{-/-} \) (\( \Delta 0.4kb \)) and \( Cd4(1.5k)^{F/F};Cd4-cre \) (\( \Delta 1.5kb \)) mice. (B) Statistical analysis of mean fluorescence intensities (MFI) of CD4 expression in pre-selection CD69\(^{-} \) DP thymocytes, CD69\(^{+} \) CD24\(^{+} \) CD8\(^{-} \) post-selection thymocytes, and TCR\( \beta^{hi} \) CD24\( ^{lo/-} \) CD8\(^{-} \) mature thymocytes. Data are shown as means and SD and statistical differences were tested by one-way ANOVA. N=9-15. (C) Expression of ThPOK, Runx1 and Runx3 proteins in CD4\(^{+} \) CD8\(^{-} \) and CD8\(^{+} \) T cells from \( \Delta 1.5kb \) and control B6 mice. Whole cell extract of two independent sets of CD4\(^{+} \) CD8\(^{-} \) and CD8\(^{+} \) T cells from \( \Delta 1.5kb \) and control B6 mice was separated by SDS-PAGE and subjected to immunoblotting. Anti-\( \beta \) tubulin antibody was used to measure loading. (D) IL-2 and IFN\( \gamma \) expression in CD4\(^{+} \) CD8\(^{-} \) and CD8\(^{+} \) T cells cultured under non-polarizing conditions. Percentages of IL-2\(^{+} \) and IFN\( \gamma \)\(^{+} \) cells are shown. Data represent three experiments with similar results.
Supplementary Fig. 3. Comparison of CD4 expression levels and T cell development in \( Cd4(1.5k)^{+/+} \)\;Cd4-cre mice and \( Cd4(1.5k)^{+/+} \)\;Vav1-cre mice.

(A) CD4 and CD8 expression in developing T cells from age-matched B6, \( Cd4(1.5k)^{+/+} \)\;Cd4-cre mice and \( Cd4(1.5k)^{+/+} \)\;Vav1-cre mice was analyzed as in Fig. 2. Percentages of gated populations in representative samples are shown. (B) Statistical analysis of numbers of CD4\(^+\)CD8\(^-\) and CD8\(^+\) mature thymocytes, and CD4\(^+\) CD8\(^-\) and CD8\(^+\) T cells in the spleen and CD4, and CD4/CD8 ratios. N=2 (B6), 4 (Cd4-cre), and 6 (Vav1-cre). Statistical differences were tested by one-way ANOVA.