Immunity
Article

RUNX Transcription Factor-Mediated Association of *Cd4* and *Cd8* Enables Coordinate Gene Regulation

Amélie Collins,[1] Susannah L. Hewitt,[2] Julie Chaumeil,[2] MacLean Sellars,[1] Mariann Micsinai,[3,4,5] Jeanne Allinne,[2] Fabio Parisi,[5] Elphège P. Nora,[6] Dan J. Bolland,[7] Anne E. Corcoran,[7] Yuval Kluger,[5] Remy Bosselut,[8] Wilfried Ellmeier,[9] Mark M.W. Chong,[1,12] Dan R. Littman,[1,10] and Jane A. Skok[2,11,*]
[1]Molecular Pathogenesis Program, The Kimmel Center for Biology and Medicine of the Skirball Institute
[2]Department of Pathology
[3]New York University Center for Health Informatics and Bioinformatics
[4]NYU Cancer Institute
New York University School of Medicine, 550 First Avenue, New York, NY 10016, USA
[5]Department of Pathology and Yale Cancer Center, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520, USA
[6]Institut Curie, CNRS UMR3215, INSERM U934, 75724 Paris Cedex 05, France
[7]The Babraham Institute, Babraham Research Campus, Cambridge, CB22 3AT, UK
[8]Laboratory of Immune Cell Biology, Center for Cancer Research (CCR), NCI, NIH, Bethesda, MD 20892-4259, USA
[9]Division of Immunobiology, Institute of Immunology, Medical University of Vienna, Lazarettgasse 19, A-1090 Vienna, Austria
[10]Howard Hughes Medical Institute
[11]Division of Infection and Immunity, The Department of Immunology and Molecular Pathology, University College London, London W1T 4JF, UK
[12]Present address: The Walter and Eliza Hall Institute, Parkville, VIC 3052, Australia
*Correspondence: jane.skok@med.nyu.edu
DOI 10.1016/j.immuni.2011.03.004

SUMMARY

T cell fate is associated with mutually exclusive expression of CD4 or CD8 in helper and cytotoxic T cells, respectively. How expression of one locus is temporally coordinated with repression of the other has been a long-standing enigma, though we know RUNX transcription factors activate the *Cd8* locus, silence the *Cd4* locus, and repress the *Zbtb7b* locus (encoding the transcription factor ThPOK), which is required for CD4 expression. Here we found that nuclear organization was altered by interplay among members of this transcription factor circuitry: RUNX binding mediated association of *Cd4* and *Cd8* whereas ThPOK binding kept the loci apart. Moreover, targeted deletions within *Cd4* modulated CD8 expression and pericentromeric repositioning of *Cd8*. Communication between *Cd4* and *Cd8* thus appears to enable long-range epigenetic regulation to ensure that expression of one excludes the other in mature CD4 or CD8 single-positive (SP) cells.

INTRODUCTION

Cell fate is determined by complex patterns of gene expression that are often mediated by a surprisingly limited number of transcription factors. Within a particular lineage, key factors can both upregulate and repress the expression of different target genes, which can number in the hundreds and be scattered throughout the genome. How are these activities coordinated? Given that tissue-specific expression profiles can be accompanied by tissue-specific patterns of locus conformation (Roldán et al., 2005; Sayegh et al., 2005; Skok et al., 2007) or nuclear location (near the nuclear periphery, pericentromeric heterochromatin [Brown et al., 1999], or within a chromosome territory [Chambeyron and Bickmore, 2004]), it is reasonable to ask whether higher-order nuclear organization might be involved in coordinating this simultaneous expression and repression.

Lymphocyte development provides an attractive model system for investigating whether there is a correlation between cell fate decisions and the spatial organization of the nucleus, because developmental stages and effector cell functions are clearly differentiated by the expression of cell surface glycoproteins that mark lineage commitment (and transcription of whose loci must therefore be carefully orchestrated). T lymphocytes express both CD4 and CD8 during development, but mature helper T cells express only CD4 protein while mature cytotoxic T cells express only CD8 (Kioussis and Ellmeier, 2002). Both populations of T cells arise from common thymic precursors that are propelled through a series of developmental stages by recombination of variable, diversity, and joining gene segments (V(D)J) that eventually form a unique antigen receptor. Recombination begins at the earliest stage of development in double-negative (DN) thymocytes, which express neither CD4 nor CD8. Productive V(D)J rearrangement of one allele leads to assembly and expression of the pre-T cell receptor (pre-TCR) on the surface of the cell; signaling through the pre-TCR promotes differentiation to the double-positive (DP) stage, in which the cells express both CD4 and CD8. DP cells then enter a transitional stage during which CD8 expression diminishes ($CD4^+CD8^{lo}$) before finally becoming either $CD4^+$ helper T cells or $CD8^+$ cytotoxic T cells (sometimes referred to as CD4 or CD8 single-positive [SP] cells, respectively). The CD4 and CD8 coreceptors are regulated to ensure mutually exclusive expression (and complementary repression).

How is this coregulation achieved? Two lines of evidence led us to suspect that nuclear organization might play a role. First, it is increasingly apparent that transcriptional status is related to chromosomal positioning (Chubb et al., 2006; Iborra et al., 1996; Osborne et al., 2004; Ragoczy et al., 2006). Second, members of the RUNX family of transcription factors are important in governing cell fate decisions in developing T lymphocytes (Collins et al., 2009)—and they are known to have a role in nuclear organization as well (Stein et al., 2007). RUNX1 and RUNX3, which are expressed at different stages of T cell development, both activate and repress expression of the *Cd4* and *Cd8* loci in a complementary fashion. How such exquisite control is achieved, however, has been difficult to ascertain with molecular genetic and biochemical approaches.

To test the notion that higher-order nuclear organization might facilitate epigenetic regulation of these loci, we used 3-dimensional DNA fluorescent in situ hybridization (FISH) to examine the interplay between the *Cd4* and *Cd8* loci during T cell development in wild-type mice and a variety of mutant lines.

RESULTS

Cd4 and *Cd8* Associate with One Another in CD8-Expressing Murine Cells

Cd4 and *Cd8* are located on chromosome 6 in the mouse, separated by a distance of 53.3 megabases (Mb). We used the two BAC probes RP23-121J20 and RP23-139M18, which cover the *Cd4* and *Cd8* loci, respectively, to follow the nuclear localization of these loci during T cell development (Figure S1A available online). 3D DNA FISH and confocal microscopy were carried out as previously described (Roldán et al., 2005) in sorted thymocyte populations from wild-type mice (Figure S1B). It is important to point out that, in our analyses, we sorted cells in different stages of thymocyte development by expression of CD4 and CD8 as well as a number of other developmental markers (Figure S1C). We measured the distance between the center of mass of the *Cd4* and *Cd8* signals in individual cells by using Image J software (Figure 1A). Interallelic distances were displayed as cumulative frequency curves. A left shift in the cumulative frequency curve indicates closer distances, as shown by the distribution. The statistical significance of the difference between distributions was calculated by the two-sample Kolmogorov-Smirnov (KS) test (Figure 1B).

Two controls were used for these experiments: we measured the distance between these same loci in a different cell type, namely B cells, and we measured the distance between two different loci, *Tcrb* and *Lrig1*, which are also located on chromosome 6 and separated by a similar distance (52.9 Mb) (Figure S1A). We observed significantly reduced association between *Cd4* and *Cd8* alleles in splenic B cells compared to DP cells ($p = 1.67e-11$) (Figure 1B). To examine the control loci *Tcrb* and *Lrig1*, we used the two BAC probes RP24-365F23 and RP23-148M10, respectively (Figure S1A). Association between *Tcrb* and *Lrig1* was significantly lower than between *Cd4* and *Cd8* in DP cells (Figure 1B; $p = 2.44e-10$). We used the distribution of the frequency of association of *Cd4* and *Cd8* in a control cell type (splenic B cells) and of *Tcrb* and *Lrig1* in DP cells to provide the background measurement of association against which the distribution in developing thymocytes could be compared.

At the earliest (DN) stage of development, when T cells express neither CD4 nor CD8, we found significantly decreased association of *Cd4* and *Cd8* compared to DP T cells (Figure 1C; $p = 3.37e-14$). As DP cells differentiate to the $CD4^+CD8^{lo}$ transitional stage, the loci dissociated somewhat ($p = 3.12e-07$), but *Cd4* and *Cd8* achieved close association again in CD8 SP cells. In CD4 SP thymocytes, the two loci moved farther apart than in the transitional $CD4^+CD8^{lo}$ stage and were significantly more separated compared to CD8 SP cells ($p = 1.43e-10$). *Cd4* and *Cd8* association therefore correlated well with CD8 expression, whether the cells were isolated from thymus, peripheral spleen, or lymph node (data not shown).

In the same subsets of sorted thymocytes, we examined the positions of the *Cd4* and *Cd8* loci relative to pericentromeric heterochromatin (PCH), a repressive subcompartment of the nucleus. This was carried out as described previously (Merkenschlager et al., 2004), with a labeled γ-satellite repeat probe to identify PCH regions. Association of *Cd4* and *Cd8* was scored if the signals were juxtaposed or overlapping with PCH. At the DN stage, approximately 50% of *Cd4* and 30% of *Cd8* alleles were associated with PCH (Figure S1D and Table S1). In DP cells, which express both proteins, ~25% of *Cd4* and ~35% of *Cd8* alleles were located at PCH. In $CD4^+CD8^{lo}$ and CD4 SP cells, a large proportion of *Cd8* alleles were repositioned to PCH (up to 70%), consistent with its diminished expression. Likewise, in CD8 SP cells, ~65% of *Cd4* alleles were repositioned to pericentromeric regions.

Taken together these data indicate that *Cd4* and *Cd8* associate specifically in T lineage cells (in contrast to B cells) and that the greatest degree of close interaction occurs in DP and CD8 SP cells. Furthermore, in agreement with what has been published, we found that repositioning of each locus to PCH inversely correlated with the expression of the coreceptor at that developmental stage (Delaire et al., 2004; Merkenschlager et al., 2004).

Cd4 and *Cd8* Association Occurs Predominantly in *cis*

The association we observed between the *Cd4* and *Cd8* loci could be occurring either in *cis* (between the two alleles on the same chromosome) or in *trans* (between the two alleles on separate chromosomes). To determine which is the case, we measured the distance between the two chromosome territories and analyzed whether *Cd4* and *Cd8* were positioned closer on the same, or different, chromosomes in DP and CD8 SP sorted T cells. We used a chromosome paint that hybridizes to chromosome 6 in addition to the two BAC probes RP23-121J20 and RP23-139M18 (Figure 1D). In 40%–60% of DP or CD8 SP cells, the two chromosome 6 territories were separated by >1 μm, indicating that any interaction between *Cd4* and *Cd8* was occurring predominantly in *cis* on the same chromosome. Even when the two territories were separated by <1 μm, it was still possible to assign each *Cd4* and *Cd8* locus to its respective chromosome territory in most cells. In only a small population of cells (10% of DP cells and 2% of CD8 SP cells) was it difficult to determine which territory the loci belonged to and whether *Cd4* and *Cd8* were closer on the same or different chromosomes. Thus,

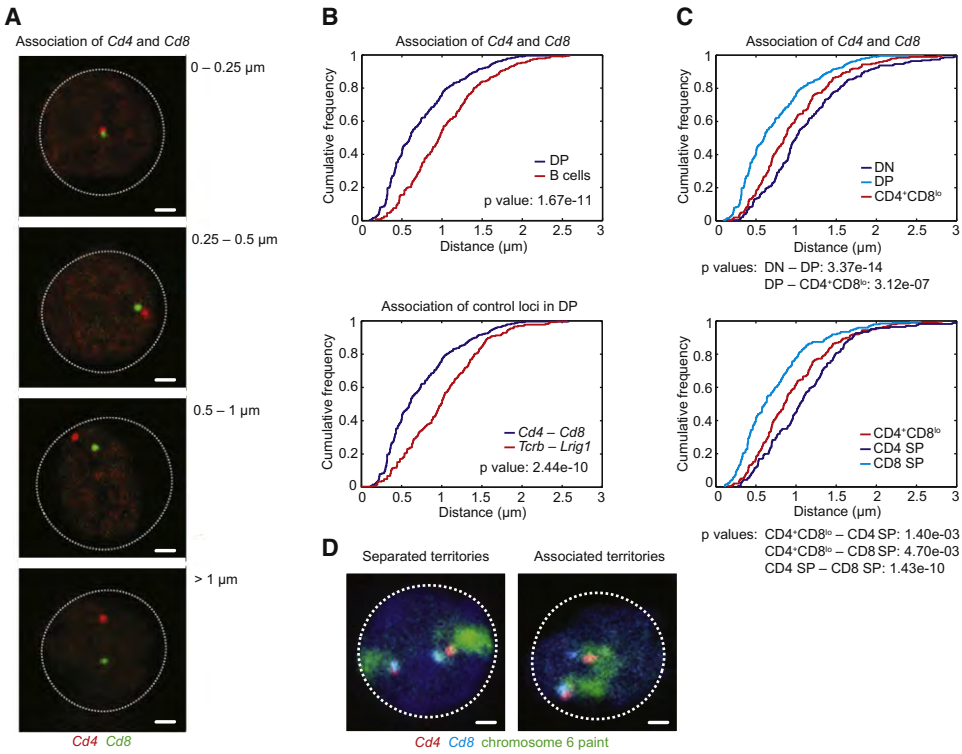

Figure 1. *Cd4* and *Cd8* Associate in Murine CD8-Expressing Cells

(A) Confocal microscopy sections showing a range of distances between the *Cd4* and *Cd8* loci. Scale bars represent 1 μm.

(B) Association of *Cd4-Cd8* in DP T cells compared to B cells (top); association of *Cd4-Cd8* compared to *Tcrb-Lrig1* in DP T cells (bottom). The separation of signals is plotted as a cumulative frequency of association. Association of *Cd4-Cd8* in DP cells was increased compared to B cells and higher than association of *Tcrb-Lrig1* in DP cells. The Kolmogorov-Smirnov test was used for statistical analysis. At least three independent experiments were performed for each data set. n = 238–264 alleles.

(C) Association of *Cd4-Cd8* in developing T cell populations and statistical analysis between specified stages. Association is increased in DP and CD8 SP cells. n = 166–238 alleles.

(D) Images of *Cd4* and *Cd8* and their individual chromosome 6 territories in DP cells.

See also Figure S1 and Table S1.

interaction between *Cd4* and *Cd8* occurs predominantly between loci located on the same chromosome.

The $E8_I$ and $E8_{II}$ Enhancers Promote *Cd8* Transcription and *Cd4-Cd8* Association

Having established that *Cd4-Cd8* association occurs in *Cd8*-expressing cells, we turned our attention to regulatory elements in the *Cd8* locus to determine how *Cd8* transcription affects the relationship between the two loci. $CD8^+$ T cells express a heterodimer of CD8α and CD8β chains that are governed by at least five enhancer elements ($E8_I$ to $E8_V$; Figure S2A) that drive expression of CD8α and CD8β in a developmentally regulated manner (Ellmeier et al., 1997, 1998; Hostert et al., 1997, 1998), although genetic analysis of these enhancer elements indicates overlapping and redundant roles in regulating CD8 expression. We first made use of mice lacking the $E8_I$ enhancer (Ellmeier et al., 1998), which is active in CD8 SP thymocytes but not DP cells (Ellmeier et al., 1997). The location of *Cd8* enhancers within the *Cd8* locus is shown in Figure S2A. DNA FISH and confocal microscopy analysis of sorted thymocyte populations from $E8_I$-deficient mice (Figure S2B) showed wild-type levels of *Cd4-Cd8* association in DP thymocytes (data not shown) but reduced association in CD8 SP cells (p = 7.19e-06 compared to wild-type CD8 SP) (Figure 2A). Consistent with this finding, *Cd8* transcription was decreased in $E8_I$-deficient CD8 SP cells but not DP cells (Figure 2B) and surface CD8 expression in all $E8_I$-deficient thymocyte subsets was 25% lower than in wild-type (Ellmeier et al., 1998). These data are consistent with the notion that *Cd8* transcription promotes association of *Cd4* and *Cd8* loci, but it is also possible that $E8_I$ mediates the association by recruiting factors to the *Cd8* locus that promote its interaction with *Cd4* in CD8 SP cells.

To explore this further, we took advantage of the variegated CD8 expression in mice doubly deficient for $E8_I$ and $E8_{II}$ (Ellmeier et al., 2002). These mice lose expression of CD8 in approximately one-third of their DP stage thymocytes while retaining wild-type amounts of surface expression in the remaining two-thirds (Figure 2C; Ellmeier et al., 2002). By gating on these populations, we were able to sort $E8_IE8_{II}$ double-mutant DP cells that are CD8-expressing and $E8_IE8_{II}$ double-mutant DP cells that have reduced CD8 expression (referred to here as $CD8^+$ $E8_IE8_{II}$ and $CD8^{lo}$ $E8_IE8_{II}$ double-mutant DP cells, respectively; Figure S2C). DNA FISH and confocal microscopy showed decreasing amounts of *Cd4-Cd8* association with reduced

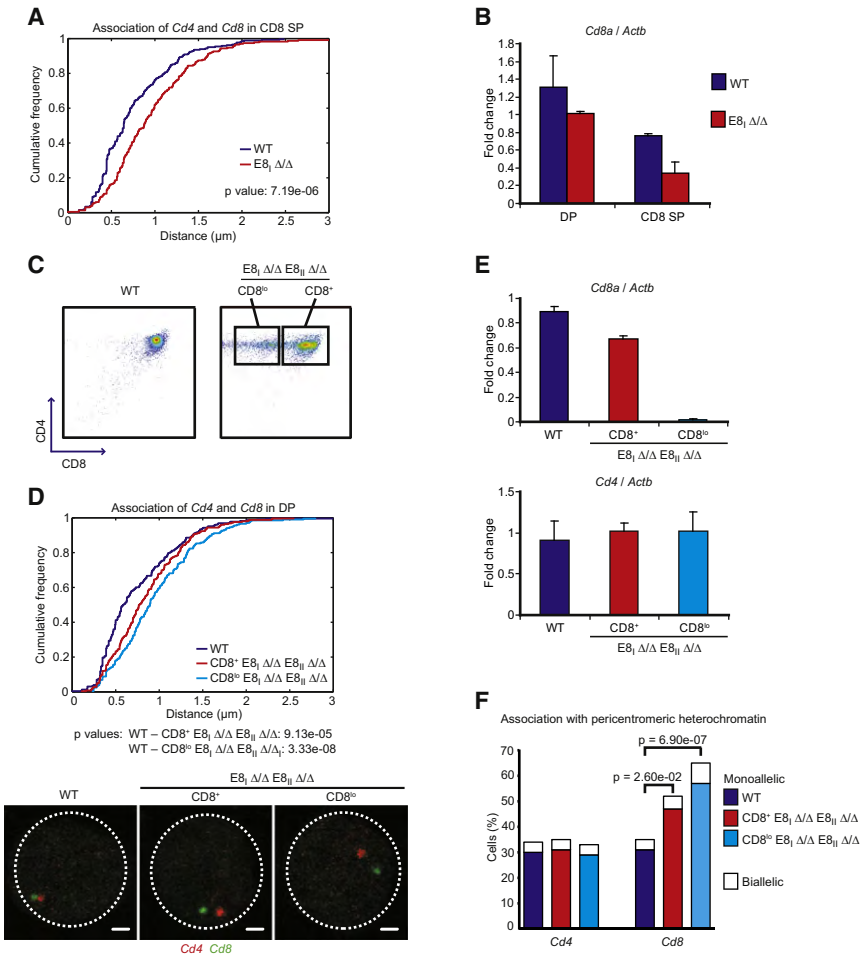

Figure 2. The $E8_I$ and $E8_{II}$ Enhancers Promote *Cd8* Transcription and *Cd4-Cd8* Association

(A) *Cd4-Cd8* association in wild-type and $E8_I$-deficient CD8 SP cells ($E8_I$ Δ/Δ). Association is decreased in $E8_I$-deficient CD8 SP compared to wild-type cells. n = 230–264 alleles.

(B) *Cd8a* RNA expression in wild-type and $E8_I$-deficient DP and CD8 SP cells. Standard error bars were calculated from three independent experiments.

(C) Flow cytometry analysis of wild-type and $E8_IE8_{II}$ double-mutant DP cells ($E8_I$ Δ/Δ $E8_{II}$ Δ/Δ) ($TCR\beta^{int}$ $CD24^+$).

(D) *Cd4-Cd8* association in wild-type and $E8_IE8_{II}$ double-mutant DP cells. Statistical analyses are between specified genotypes. Association is lower in $CD8^+$ and $CD8^{lo}$ $E8_IE8_{II}$ double-mutant than in wild-type cells. Confocal microscopy sections of *Cd4-Cd8* distances are representative of each genotype. Scale bars represent 1 μm. n = 204–248 alleles.

(E) RT-PCR analysis of *Cd8a* (top) or *Cd4* (bottom) expression in wild-type and $E8_IE8_{II}$ double-mutant DP cells. Standard error bars were calculated from two independent experiments.

(F) *Cd4* or *Cd8* association with pericentromeric heterochromatin in wild-type and $E8_IE8_{II}$ double-mutant DP cells. *Cd8* recruitment is higher in double-mutant than in wild-type control cells.

See also Figure S2 and Table S2.

CD8 expression: $CD8^+$ $E8_IE8_{II}$ double-mutant DP cells showed significantly less *Cd4-Cd8* association than did wild-type (p = 9.13e-05) and $CD8^{lo}$ $E8_IE8_{II}$ double-mutant DP cells showed even lower levels of *Cd4-Cd8* association (p = 3.33e-08 compared to wild-type) (Figure 2D). Consistent with these results, RT-PCR analysis demonstrated that *Cd8* transcription was lower than wild-type in $CD8^+$ $E8_IE8_{II}$ and almost abolished in $CD8^{lo}$ $E8_IE8_{II}$ double-mutant DP cells (Figure 2E).

Repositioning of *Cd8* to PCH was probably affected both by deletion of these enhancer elements and by the reduction in transcription. We observed increased positioning of *Cd8* to PCH in the $CD8^+$ $E8_IE8_{II}$ double-mutant DP cells; about 52% of the double-mutant cells had at least one allele associated with PCH in $CD8^+$ DP, versus 35% in wild-type DP cells. This repositioning was even greater (65%) in $CD8^{lo}$ $E8_IE8_{II}$ double-mutant DP cells (p = 2.60e-02 for $CD8^+$ $E8_IE8_{II}$ double-mutant DP compared to wild-type; p = 6.90e-07 for $CD8^{lo}$ $E8_IE8_{II}$ double-mutant DP compared to wild-type; Figure 2F; Table S2). Pericentromeric localization of *Cd8* correlates with epigenetic differences between $CD8^{lo}$ $E8_IE8_{II}$ double-mutant DP cells and $CD8^+$ $E8_IE8_{II}$ double-mutant DP because the *Cd8* locus in the $CD8^{lo}$ cells has an epigenetic "off" state (Bilic et al., 2006).

Decreased *Cd8* transcription therefore correlates with diminished association of *Cd4* and *Cd8* and increased repositioning of *Cd8* alleles to PCH. It is not clear, however, whether *Cd8* transcription is a cause or a consequence of the euchromatic location of *Cd8* and the increased association of *Cd4-Cd8.* Both of these effects could be mediated by the presence of *trans*-acting factors recruited to the *Cd8* locus by $E8_I$ and $E8_{II}$.

Cd4-Cd8 Association Requires the RUNX Binding Partner CBFβ

To determine whether *trans*-acting proteins are involved in mediating the *Cd4-Cd8* association, we focused on members of the RUNX family, which are known to bind both the *Cd4* and *Cd8* loci and have an important role in governing cell fate decisions in developing T lymphocytes (Collins et al., 2009). RUNX1 and RUNX3 are expressed at different stages of T cell development: RUNX1 binds the *Cd4* silencer element in DN cells to suppress *Cd4* expression (Taniuchi et al., 2002a; Zou et al., 2001), and loss of RUNX1 or CBFβ (which stabilizes the interaction of RUNX proteins with DNA) or deletion of the *Cd4* silencer allows *Cd4* expression in DN cells (Leung et al., 2001; Taniuchi et al., 2002b). RUNX3 expression is activated at later stages of T cell development, and in addition to binding to the *Cd4* silencer to prevent *Cd4* derepression, it has a crucial role in activating *Cd8* expression in transitional $CD4^+CD8^{lo}$ and CD8 SP cells (Egawa et al., 2007). RUNX proteins bind along the *Cd8* locus, most prominently to $E8_I$ (specifically in CD8 SP cells) and to $E8_{II}$ in DP cells as well as $E8_{IV}$ in all thymocytes (Sato et al., 2005). Thus, RUNX1 and RUNX3 simultaneously

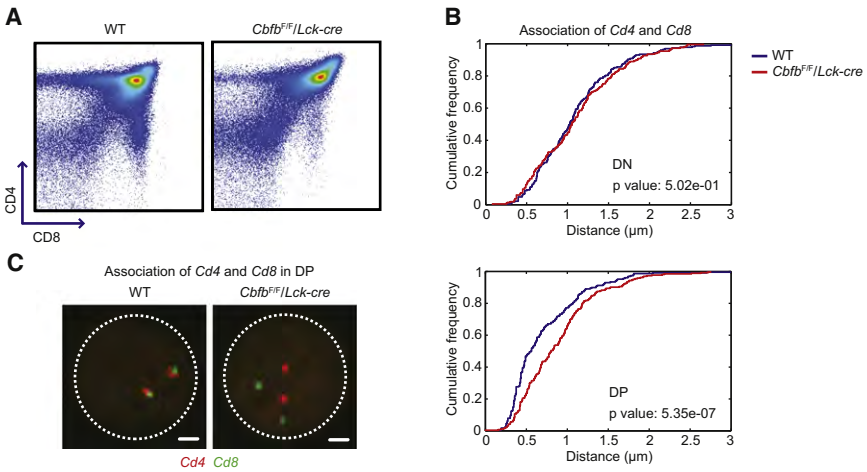

Figure 3. *Cd4-Cd8* Association Requires the RUNX Binding Partner CBFβ

(A) Flow cytometry analysis of wild-type and CBFβ-deficient thymocytes (conditional *Cbfb*$^{F/F}$ crossed to *Lck-cre*).

(B) *Cd4-Cd8* association in wild-type and CBFβ-deficient DN and DP T cells, including statistical analysis. Association is lower in CBFβ-deficient DP cells than in wild-type counterparts. n = 228–264 alleles.

(C) Confocal microscopy sections of *Cd4-Cd8* distances representative of wild-type or CBFβ-deficient DP cells. Scale bars represent 1 μm.

See also Figure S3.

regulate expression of the *Cd4* and *Cd8* loci in an opposite manner.

To test the hypothesis that RUNX proteins are involved in bringing the *Cd4* and *Cd8* loci together, we used conditional *Cbfb*-deleted mice in which the gene encoding CBFβ, the requisite heterodimeric binding partner of all RUNX proteins, is conditionally deleted in all thymocytes from the DN stage onward by crossing to *Lck-cre* mice, which permits transition to the DP stage but results in blocked T cell development beyond the DP stage (Figure 3A; Egawa et al., 2007). Our DNA FISH and confocal analyses of sorted thymocyte populations from CBFβ-deficient mice (Figure S3) revealed that *Cd4-Cd8* come into close contact at a lower frequency in DP cells from these mice (p = 5.35e-07; Figures 3B and 3C). This indicates a role for RUNX proteins in mediating association between the two loci.

ThPOK Inhibits the Association of *Cd4* and *Cd8*

The zinc finger transcription factor ThPOK, which has been shown to be required for CD4$^+$ T cell lineage commitment (He et al., 2005; Sun et al., 2005), binds to the *Cd4* silencer to prevent CD4 silencing in CD4-fated thymocytes (Muroi et al., 2008). In CD4-fated cells, ThPOK expression is increased from the basal post-positive selection level, and ThPOK binds to both the *Zbtb7b* (gene encoding ThPOK) silencer and the *Cd4* silencer, where it is thought to antagonize RUNX function and prevent the *Zbtb7b* and *Cd4* loci from being silenced (Muroi et al., 2008; Wildt et al., 2007). ThPOK has also been implicated in repressing *Cd8* expression (Jenkinson et al., 2007), and peripheral CD8$^+$ T cells transduced with a retroviral vector expressing *Zbtb7b* have significantly lower *Cd8* transcription than either empty vector-transduced CD8$^+$ T cells or those transduced with a *Zbtb7b* retrovirus carrying the HD mutation (which leads to a defect in the generation of CD4$^+$ T helper cells) (Figure 4A; Dave et al., 1998). Furthermore, ThPOK-deficient mice show increased expression of RUNX3 in CD4$^+$CD8^{lo} transitional cells (Egawa and Littman, 2008), which could exert an effect on *Cd4-Cd8* association.

We generated *Zbtb7b*$^{hd/hd}$ mice (Figures S4A–S4D), sorted the few remaining CD4 SP thymocytes (Figure 4B; Figure S4E), and used a RUNX3-specific antibody for immunofluorescence. Wild-type CD4 SP cells expressed very little RUNX3 protein compared to wild-type CD8 SP cells, but *Zbtb7b*$^{hd/hd}$ CD4 SP cells expressed levels of RUNX3 equivalent to wild-type CD8 SP cells (Figure 4C). The increase in RUNX3 expression in CD4 SP cells from *Zbtb7b*$^{hd/hd}$ mice significantly increased interaction between *Cd4* and *Cd8* (p = 3.15e-03 compared to wild-type CD4 SP cells) (Figure 4D). Furthermore, we observed a significant rise in the percentage of *Cd4* alleles positioned at PCH (from 40% in wild-type CD4 SP cells to almost 70% in *Zbtb7b*$^{hd/hd}$ CD4 SP cells, p = 1.14e-05; Figure 4E; Table S3). In line with our previous observations, the increased frequency in *Cd4-Cd8* association was accompanied by an increase in *Cd8* transcription (Figure 4F). These data suggest that one of the functions of ThPOK could be to inhibit the reassociation of the *Cd4* and *Cd8* loci in CD4-fated CD4$^+$CD8^{lo} and CD4 SP thymocytes, thereby preventing *Cd4* silencing.

To test the idea that ThPOK could separate *Cd4* and *Cd8*, we compared the frequency of association of these loci in DP cells from wild-type and ThPOK transgenic mice (Sun et al., 2005). These mice express a wild-type form of the protein by using human CD2-based regulatory elements that drive expression as early as the DP stage of development (where the endogenous locus is not normally transcribed). Although the presence of the transgene does not substantially alter the total number of thymocytes, it directs cells toward the CD4 lineage and impairs CD8 development; there are virtually no CD8 SP cells in these mice (Sun et al., 2005). We found a significant decrease in the frequency of *Cd4-Cd8* association in DP cells where ThPOK is prematurely expressed (p = 1.40e-08) (Figure 4G and for sort strategy see Figure S4F). Together these experiments indicate that ThPOK negatively regulates association of *Cd4* and *Cd8* as well as commitment to the CD8 lineage.

The *Cd4* Proximal Enhancer Inhibits *Cd4-Cd8* Association after Positive Selection

The experiments we have described above indicate that the transcription factor RUNX could bring *Cd4* and *Cd8* together to streamline their regulation (Schoenfelder et al., 2010). It is also possible that *Cd4* and *Cd8* could exert a more direct influence over each other. If so, alterations of key regulatory elements within the *Cd4* locus would translate into changes in *Cd8* regulation. To address this question, we made use of gene-targeted

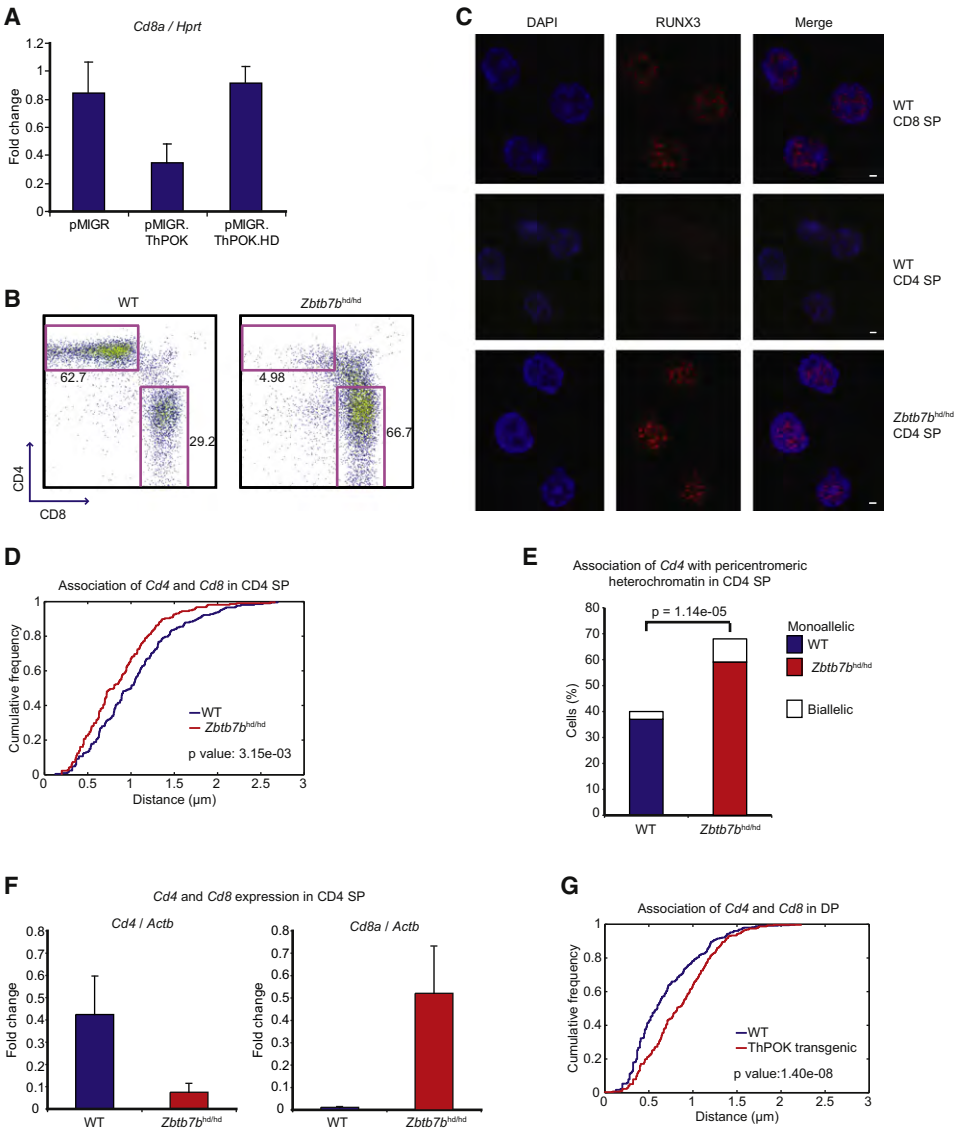

Figure 4. ThPOK Inhibits *Cd4-Cd8* Association

(A) *Cd8a* RNA expression in peripheral CD8$^+$ T cells transduced with empty pMIGR, pMIGR.ThPOK, or pMIGR.ThPOK.HD.

(B) Flow cytometry analysis of wild-type and *Zbtb7b*$^{hd/hd}$ mature SP cells.

(C) RUNX3 staining in wild-type CD8 cells and wild-type or *Zbtb7b*$^{hd/hd}$ CD4 SP cells. Scale bars represent 1 μm.

(D) *Cd4-Cd8* association in wild-type and *Zbtb7b*$^{hd/hd}$ CD4 SP cells. Association is higher in *Zbtb7b*$^{hd/hd}$ CD4 SP than in wild-type cells. n = 206–218 alleles.

(E) *Cd4* recruitment to pericentromeric heterochromatin in wild-type and *Zbtb7b*$^{hd/hd}$ CD4 SP cells. Recruitment is higher in *Zbtb7b*$^{hd/hd}$ cells.

(F) RT-PCR analysis of *Cd4* or *Cd8a* expression in wild-type and *Zbtb7b*$^{hd/hd}$ CD4 SP cells. Standard error bars were calculated from three independent experiments.

(G) *Cd4-Cd8* association in wild-type and ThPOK transgenic DP cells. *Cd4-Cd8* association is lower in ThPOK transgenic DP cells. n = 316–356 alleles.

See also Figure S4 and Table S3.

mice. *Cd4* expression is regulated by a silencer element and at least one stage-specific enhancer element (Chong et al., 2010; Kioussis and Ellmeier, 2002). The proximal enhancer *Cd4* PE, located 13 Kb upstream of the *Cd4* start site, is absolutely required for transcription, and therefore expression, of *Cd4* in DP thymocytes (Chong et al., 2010). The position of this enhancer is diagrammed in Figure S5A. After positive selection in *Cd4* proximal enhancer (PE)-deficient mice, CD4-expressing single-positive thymocytes and CD4$^+$ peripheral T cells were detected, albeit at reduced numbers, and levels of CD4 expression were comparable to wild-type mice, suggesting that one or more putative enhancer elements rescue *Cd4* expression (Figure 5A and data not shown). DNA FISH and confocal microscopy analysis of sorted thymocyte populations from *Cd4* PE-deficient mice (Figure S2B) revealed that the *Cd4* PE, and therefore *Cd4* transcription, is not required for either the *Cd4-Cd8* association at the DP stage or for the repositioning away from PCH, because the degree of *Cd4-Cd8* association and

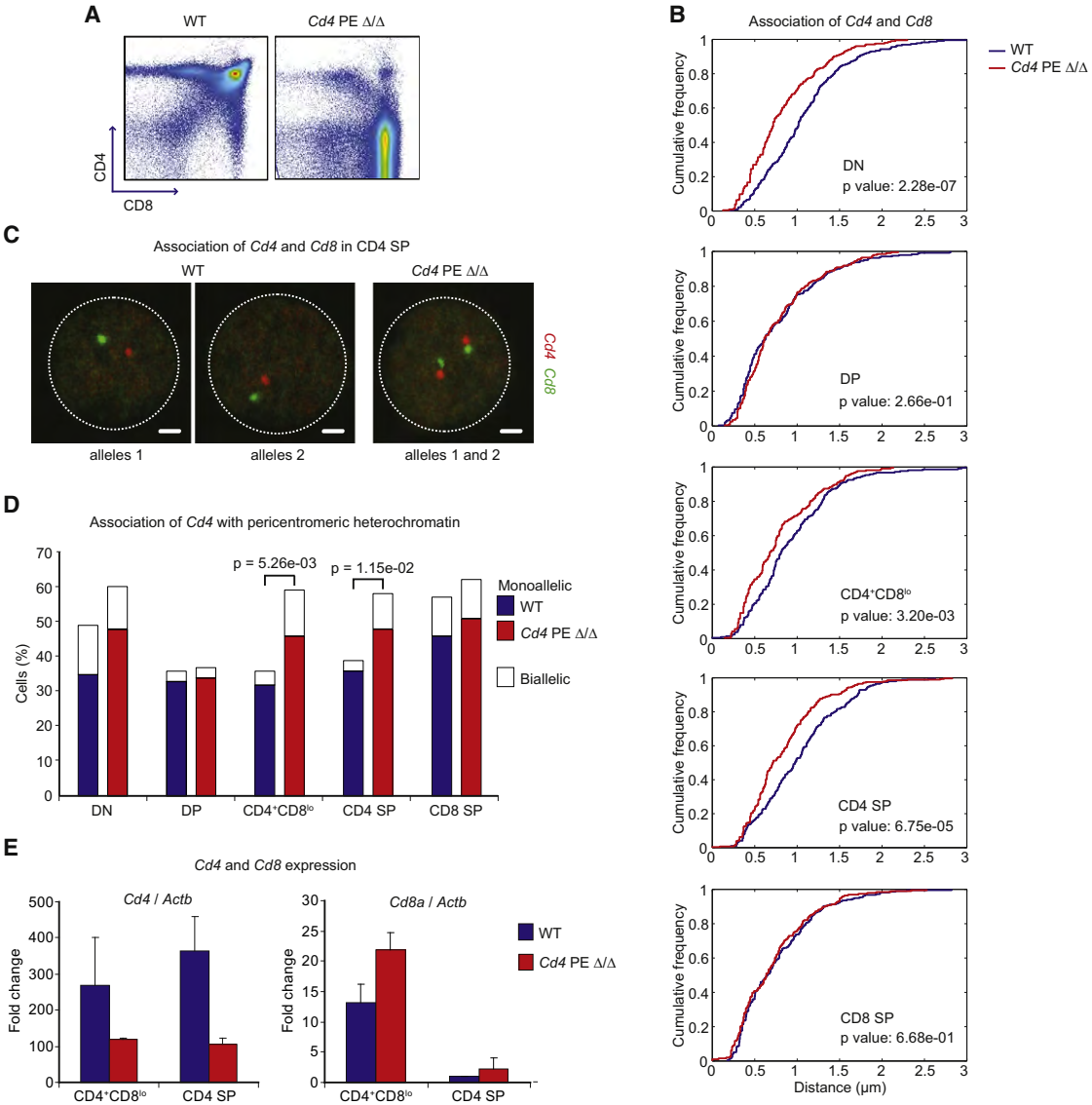

Figure 5. The *Cd4* Proximal Enhancer Inhibits *Cd4-Cd8* Association

(A) Flow cytometry analysis of wild-type and *Cd4* PE-deficient thymocytes (*Cd4* PE Δ/Δ).

(B) *Cd4-Cd8* association in wild-type and *Cd4* PE-deficient cells, including statistical analysis. Association is higher in *Cd4* PE-deficient DN, $CD4^+CD8^{lo}$, and CD4 SP cells than in wild-type cells. n = 196–286 alleles.

(C) Confocal microscopy sections of *Cd4-Cd8* distances representative of each genotype. Scale bars represent 1 μm.

(D) Recruitment of *Cd4* to pericentromeric heterochromatin in wild-type and *Cd4* PE-deficient cells. Recruitment is higher in *Cd4* PE-deficient DN, $CD4^+CD8^{lo}$ and CD4 SP than in wild-type cells.

(E) RT-PCR analysis of *Cd4* or *Cd8a* expression in wild-type and *Cd4* PE-deficient cells. Standard error bars were calculated from three independent experiments. See also Figure S5 and Table S4.

pericentromeric localization were comparable to wild-type (Figures 5B–5D; Table S4).

In contrast to wild-type cells, however, *Cd4-Cd8* association in *Cd4* PE-deficient mice occurred at a higher frequency in DN cells (p = 2.28e-07 compared to wild-type controls) and remained high in both $CD4^+CD8^{lo}$ and CD4 SP cells after positive selection (p = 3.20e-03 and p = 6.75e-05 for $CD4^+CD8^{lo}$ and CD4 SP cells, respectively, compared to the appropriate wild-type controls) (Figure 5B). Deletion of the *Cd4* PE also increased the frequency with which the *Cd4* locus relocated to PCH beyond the DP stage; in both $CD4^+CD8^{lo}$ and CD4 SP cells from *Cd4* PE-deficient mice, *Cd4* association with PCH reached the same levels as in CD8 SP cells (59% in *Cd4* PE-deficient cells compared to 36% in wild-type $CD4^+CD8^{lo}$ cells, p = 5.26e-03; 58% in *Cd4* PE-deficient cells compared to 39% in wild-type CD4 SP cells, p = 1.15e-02; Figure 5D; Table S4). In order to correlate these positional changes with the transcriptional state of each locus, we set aside a subset of each sorted thymocyte

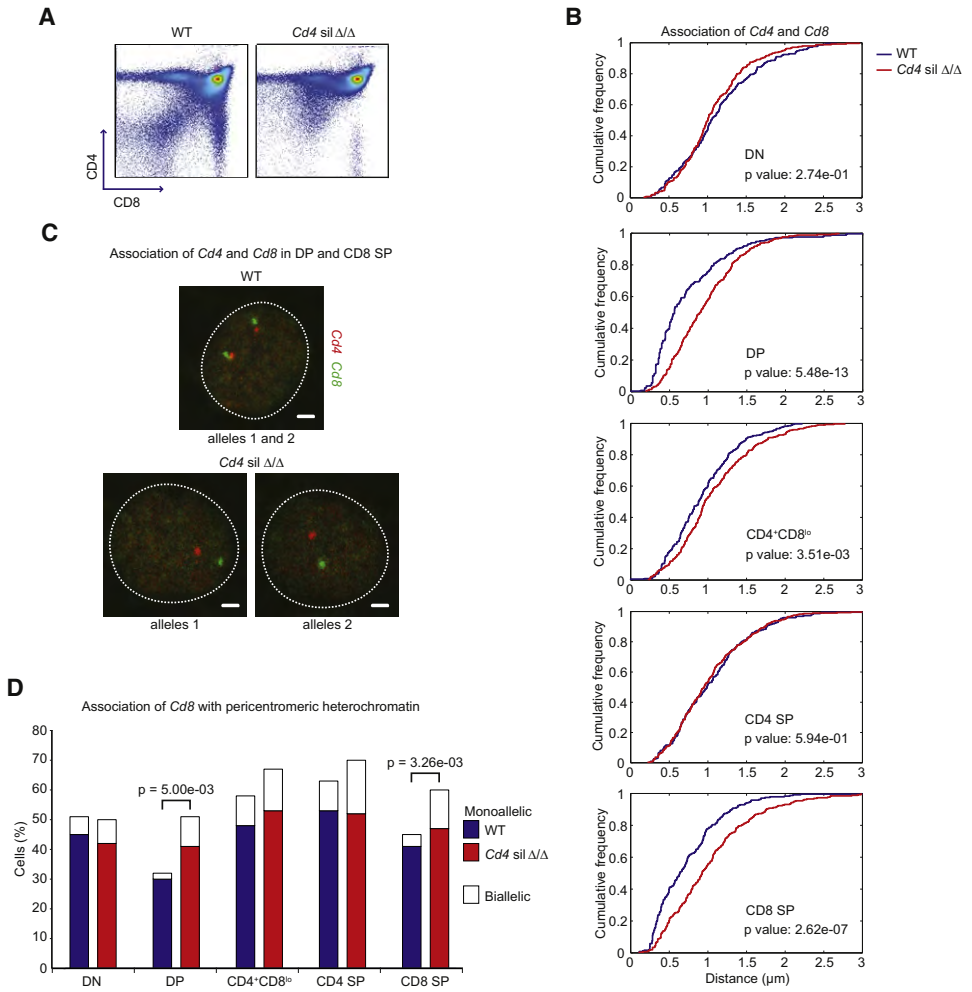

Figure 6. The *Cd4* Silencer Mediates *Cd4-Cd8* Association

(A) Flow cytometry analysis of wild-type and *Cd4* sil-deficient thymocytes (*Cd4* sil Δ/Δ).

(B) *Cd4-Cd8* association in wild-type and *Cd4* sil-deficient cells, including statistical analysis. Association is lower in *Cd4* sil-deficient DP, $CD4^+CD8^{lo}$, and CD8 SP than in wild-type cells. n = 210–340 alleles.

(C) Confocal microscopy sections of *Cd4-Cd8* distances representative of each genotype. Scale bars represent 1 μm.

(D) *Cd8* recruitment to pericentromeric heterochromatin in wild-type and *Cd4* sil-deficient cells. Recruitment is higher in *Cd4* sil-deficient DP and CD8 SP cells than in wild-type cells.

See also Figure S6 and Table S5.

population for real-time RT-PCR analysis. *Cd4* transcription was virtually abolished in DP cells from *Cd4* PE-deficient mice (data not shown) and substantially reduced in $CD4^+CD8^{lo}$ and CD4 SP cells (Figure 5E), consistent with the surface expression of CD4 on these thymocyte subsets. These data demonstrate that *Cd4* transcription is not required for *Cd4* to associate with *Cd8* at the DP stage and further suggest that, in the absence of robust transcription after positive selection, the *Cd4* locus remains associated with the *Cd8* locus. *Cd4-Cd8* pairs were more frequently located at PCH, with *Cd4* positioned close to these regions, whereas *Cd8* remained euchromatic, i.e., the two loci were not equivalently associated with this repressive compartment. Furthermore, *Cd8* expression was slightly increased in DP and $CD4^+CD8^{lo}$ cells. These results are consistent with results in wild-type cells above: association of the two loci correlates with CD8 expression.

The *Cd4* Silencer Mediates *Cd4-Cd8* Association

To explore the role of the *Cd4* silencer on *Cd4-Cd8* association and the coordinate regulation of the two loci, we next analyzed the organization of these genes in sorted thymocyte populations from wild-type and *Cd4* silencer-deficient mice. The position of the silencer within the *Cd4* locus is shown in Figure S6A (Sawada et al., 1994). Germline deletion of the *Cd4* silencer (sil) allows *Cd4* derepression in DN thymocytes and CD8 lineage T cells (Taniuchi et al., 2002b); flow cytometry analysis shows a lack of non-CD4-expressing cells in both these populations (Figure 6A).

DNA FISH was performed on sorted thymocyte populations from wild-type and *Cd4* sil-deficient mice (Figure S6B) as described previously, and cells were analyzed by confocal microscopy. Surprisingly, the *Cd4-Cd8* association in *Cd4* sil-deficient DP cells did not increase beyond the level observed

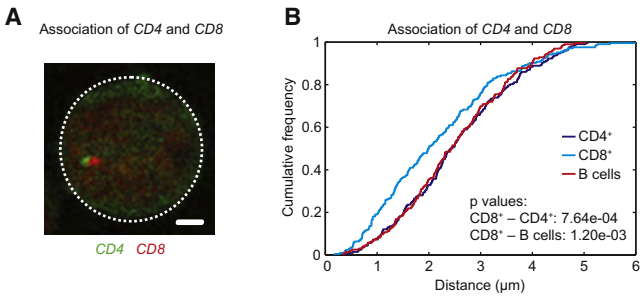

Figure 7. *CD4* and *CD8* Also Associate in Human CD8-Expressing Cells

(A) 3D DNA FISH on human sorted $CD8^+$ T cells, $CD4^+$ T cells, and B cells.

(B) *CD4-CD8* association in B and T cells, including statistical analysis between specified cell types. Association in $CD8^+$ T cells is increased compared to $CD4^+$ T and B cells. n = 196–206 alleles.

See also Figure S7.

in the DN thymocyte population and remained low at all subsequent stages of development (p = 5.48e-13 in DP cells, p = 3.51e-03 in $CD4^+CD8^{lo}$ cells, and p = 2.62e-07 in CD8 SP cells, compared to the appropriate wild-type controls; Figure 6B). Confocal sections showing decreased association of *Cd4* and *Cd8* in DP and CD8 SP cells from *Cd4* sil-deficient mice are shown in Figure 6C. These data indicate that the *Cd4* silencer region is required to mediate the close association of *Cd4* and *Cd8* in both DP and CD8 SP thymocytes.

Absence of the *Cd4* silencer did not appear to affect localization of *Cd4* at PCH, but, surprisingly, *Cd8* alleles were significantly repositioned to PCH at the DP stage (51% in *Cd4* sil-deficient cells compared to 32% in wild-type DP cells, p = 5.00e-03) and subsequent stages of development, most notably in CD8 SP cells (60% in *Cd4* sil-deficient cells compared to 45% in wild-type CD8 SP cells, p = 3.26e-03; Figure 6D; Table S5). Loss of a regulatory element on the *Cd4* locus thus results in a long-range epigenetic effect on the *Cd8* locus. Consistent with results described above, the decreased association of *Cd4* and *Cd8* and the increased repositioning of *Cd8* to PCH correlated with slightly decreased *Cd8* transcription in DP and CD8 SP cells (data not shown).

CD4 and *CD8* Associate in Human Peripheral $CD8^+$ T Cells but Not Peripheral $CD4^+$ T Cells or B Cells

Unlike the murine loci, *CD4* and *CD8* are located on different chromosomes in humans. To test whether association between *CD4* and *CD8* is conserved between the two species despite this difference, we measured association between the *CD4* and *CD8* loci, which in human cells are located on chromosomes 12 and 2, respectively. If colocalization serves an important role in coordinating the expression of these coreceptors, we would expect that the two loci should be associated more frequently in CD8-expressing T cells. We performed 3D DNA FISH and confocal microscopy analysis on peripheral $CD4^+$ and $CD8^+$ T cells as well as on B cells sorted from human peripheral blood cells (Figure S7A). For this experiment we used two BAC probes, RP11-101F21 and CTD-2291B5, which hybridize to *CD4* and *CD8* on chromosomes 12 and 2 (Figure 7A).

As expected (because the two loci are located on different chromosomes) *CD4-CD8* interallelic distances are much larger in human cells than in murine cells. Nonetheless, we observed closer association of *CD4* and *CD8* in human peripheral $CD8^+$ T cells than in either peripheral $CD4^+$ T cells or B cells (p = 7.64e-04 and p = 1.20e-03, respectively; Figure 7B; Table S6). These data indicate that cross-talk between the *Cd4* and *Cd8* loci is conserved between species, underscoring the importance of this relationship in regulating expression of the two loci (Figure S7B).

DISCUSSION

Recent studies have begun to reveal the complex interplay between nuclear organization, chromatin architecture, and gene expression. Several lines of evidence from the current study link *Cd4-Cd8* association with *Cd8* transcription. First, we observed that *Cd4* and *Cd8* closely associated only in wild-type thymocytes that express CD8 (DP and CD8 SP, but not DN, $CD4^+CD8^{lo}$, or CD4 SP). Second, where *Cd4-Cd8* association was decreased (i.e., in $CD8^+$ and $CD8^{lo}$ $E8_IE8_{II}$ double-mutant DP cells, $E8_I$-deficient CD8 SP cells, and *Cd4* sil-deficient DP and CD8 SP cells), *Cd8* transcription was also decreased to varying degrees. Third, when there was prolonged association between *Cd4* and *Cd8* (in *Cd4* PE-deficient $CD4^+CD8^{lo}$ cells, *Cd4* PE-deficient CD4 SP cells, and $Zbtb7b^{hd/hd}$ CD4 SP cells), *Cd8* transcription increased. Thus, the loss of a regulatory element (*Cd4* PE) in one locus (*Cd4*) can influence the transcriptional status of a distant locus (*Cd8*), presumably through their physical association. Similarly, loss of the *Cd4* sil on the *Cd4* locus exerts an influence on the *Cd8* locus, increasing the frequency with which the latter is positioned at PCH.

Beyond elucidating the genetic requirements for *Cd4-Cd8* association, we also wanted to gain insight into whether transcription factors known to be involved in T cell lineage commitment could be involved in mediating the association between *Cd4* and *Cd8*. For this, we made use of mice with a conditional $Cbfb^{F/F}$ allele crossed to *Lck-cre* to delete CBFβ at the early DN stage. We found that *Cd4-Cd8* association was substantially reduced in the CBFβ-deficient DP cells, despite equivalent levels of *Cd4* and *Cd8* transcription, suggesting that association of the two loci could occur at sites where RUNX is enriched in the nucleus. Loss of ThPOK leads to elevated expression of RUNX3 in CD4-fated thymocytes (Egawa and Littman, 2008), and overexpression of ThPOK in peripheral $CD8^+$ T cells decreases CD8 expression. We therefore predicted that loss of ThPOK might prolong the *Cd4-Cd8* association in CD4 SP cells from $Zbtb7b^{hd/hd}$ mice. This is indeed what we observed, indicating that binding of ThPOK to the *Cd4* locus could be a mechanism for keeping the two loci separate. Furthermore, the loss of *Cd4-Cd8* association in these cells was accompanied by a substantial increase in the localization of *Cd4* to pericentromeric heterochromatin and a concomitant decrease in *Cd4* transcription. Conversely premature expression of ThPOK in DP cells led to separation of *Cd4* and *Cd8*.

Is association between *Cd4* and *Cd8* a cause or consequence of *Cd8* transcription? We believe it may be both, in the same way that changes in location of loci relative to pericentromeric heterochromatin are likely to be both a cause and a consequence of

changes in gene activation and repression. As with most epigenetic correlations, this is a chicken-and-egg situation and we cannot pinpoint the initiating event.

These studies allow us to put forth the following model. The *Cd4* and *Cd8* loci come into close proximity in DP thymocytes. After positive selection, all thymocytes pass through a $CD4^+$ $CD8^{lo}$ transitional stage in which *Cd8* transcription decreases and it moves to pericentromeric regions, disrupting the *Cd4-Cd8* association. In CD4-fated cells, ThPOK binds to the *Cd4* silencer, preventing it from interacting again with the *Cd8* locus. In CD8-fated cells, RUNX3 mediates the reassociation of *Cd4* and *Cd8* by binding to the *Cd4* silencer and the *Cd8* locus, predominantly within $E8_I$. Thus, RUNX-mediated *Cd4-Cd8* association silences the *Cd4* locus, repositioning it to repressive pericentromeric heterochromatin.

Although it has been known for some time that chromosomal interactions can exert an effect on gene expression in *trans* in *Drosophila* (transvection) (Lewis, 1985) and possibly plants (paramutation) (Stam, 2009), there are still only a few instances in which association of alleles is known to exert epigenetic control in mammals. Two examples involve the pairing of homologous alleles: X inactivation (Bacher et al., 2006; Xu et al., 2006) and allelic exclusion (Hewitt et al., 2009). Heterologous association between different loci has been noted in developing B cells as well: one *immunoglobulin light chain* (*Igk*) allele transiently associates with one *immunoglobulin heavy chain* (*Igh*) allele at pericentromeric regions, inducing a change in nuclear location and a conformational change within the *Igh* locus to prevent ongoing recombination (Hewitt et al., 2008). Similarly, association of different loci has been shown to occur in T cell subsets: the *Ifng* locus interacts with the *Il4* locus just prior to commitment to either the Th1 or Th2 cell lineage, which express either IFN-γ or IL-4, respectively. The association of *Ifng* and *Il4* could facilitate the coordinate regulation of these loci in the differentiated $CD4^+$ T cell subsets (Spilianakis et al., 2005) but no *trans* acting factors that could mediate the association have been identified. Clearly this is an underexplored area of epigenetic regulation.

Our findings add to a growing body of evidence that nuclear architecture plays a dynamic role in regulating gene expression (Fraser and Bickmore, 2007). That association of *Cd4-Cd8* is conserved in both mouse and humans, despite being located on different chromosomes in the latter, underscores the importance of this mechanism for regulating CD4 and CD8 coreceptor expression. Undoubtedly, a fuller understanding of the mechanism of *Cd4-Cd8* association will yield insight into how these coreceptors are regulated during T cell development and how long-range chromosomal interactions control gene expression.

EXPERIMENTAL PROCEDURES

Mice

C57Bl/6 mice were purchased from Jackson Laboratories or Taconic. *Cd4* PE-deficient (Chong et al., 2010), *Cd4* sil-deficient (Zou et al., 2001), $Cbfb^{F/F}$ (Naoe et al., 2007), $E8_I$-deficient (Ellmeier et al., 1998), $E8_IE8_{II}$ double-mutant (Ellmeier et al., 2002), *L*ck-cre (Lee et al., 2001), and ThPOK transgenic (Sun et al., 2005) mice have previously been described. Mice were housed in SPF conditions at the Skirball animal facility at NYU School of Medicine. Experiments were performed in accordance with approved protocols for the NYU Institutional Animal Care and Usage Committee (IACUC).

Generation of $Zbtb7b^{hd/hd}$ Mice

A targeting vector was created with a neomycin selection cassette and *Zbtb7b* exons 2 and 3 mutated at position 389 from arginine to glycine (assembled from BAC RP23-126P10) and flanked by loxP sites (Figure S4A). An XbaI site was inserted for Southern blot screening. The construct was targeted into E14 129Sv embryonic stem cells. Breeding of derived mice with *EIIA-cre* mice (cre expression in early embryos) gave progeny with the mutant allele. Mice were backcrossed onto C57Bl/6 for at least four generations and screened by Southern blot and PCR (Figures S4B–S4D). Primer sequences for targeting vectors and genotyping are available upon request.

Flow Cytometry Analysis and Cell Sorting

Analyses and sorting were performed on an LSRII or FACSAria, respectively (both BD Biosciences; Figures S1–S7). Antibodies to mouse antigens were as follows: CD24 FITC (clone M1/69, BD, 1:1000 dilution), CD69 PE (H1.2F3, BD, 1:100), TCRβ APC (H57-597, eBiosciences, 1:500), CD4 Alexa Fluor 700 (GK1.5, eBioscience, 1:1000), CD8α PE-Cy7 (53-6.7, eBioscience, 1:1000), CD44 PE-Cy5.5 (IM7, eBioscience, 1:500), CD25 PE-Cy7 (PC61.5, eBioscience, 1:500), and Thy1.2 FITC (CD90.2, clone 30-H12, BD, 1:500). Antibodies to human antigens were as follows: CD4 Pacific Blue (RPA-T4, BD, 1:50), CD8a FITC (RPA-T8, eBioscience, 1:50), CD3 APC-Cy7 (UCHT1, eBioscience, 1:50), CD45RA PE (HI100, eBioscience, 1:20), CD45RO APC (UCHL1, eBioscience, 1:20), and CD19 FITC (HIB19, eBioscience, 1:50).

T and B Cell Isolation from Human Peripheral Blood Mononuclear Cells

Adult PBMCs were isolated (Manel et al., 2008) and depleted of $CD14^+$ cells (autoMACS Pro). $CD4^+$ and $CD8^+$ T cells and B cells were purified by flow cytometry as $CD3^+CD4^+CD45RA^+CD45RO^-$ or $CD3^+CD8^+CD45RA^+CD45RO^-$ and $CD3^-CD19^+$, respectively (Figure S7).

Purification of Resting Splenic B Cells

This was carried out as described (Skok et al., 2001).

Three-Dimensional DNA FISH

Sorted cells were washed in PBS, attached to glass coverslips coated with poly-L-lysine, and fixed for DNA FISH as described (Skok et al., 2001). Bacterial artificial chromosome (BAC) probes and the γ-satellite probe (Skok et al., 2001) were labeled by nick translation with ChromaTide Alexa Fluor 488-5-dUTP, 594-5-dUTP (Invitrogen), or dUTP-indodicarbocyanine (Cy5; GE Healthcare).

Cd4-Cd8 DNA FISH Combined with Chromosome 6 Paint

Cells were dropped onto poly-L-lysine coated slides, incubated in 0.075 M KCl (10 min), fixed in cold methanol/acetic acid 3:1 (2× 10 min), and dehydrated in an ethanol series. RNaseA treatment (100 μg/ml, 1 hr) and dehydration preceded denaturation (70% formamide/2× SSC, 75°C, 3 min), dehydration, and probe hybridization (overnight, 37°C, humid chamber). Slides were rinsed in 50% formamide/2× SSC (3×, 45°C) and 1× SSC (3×, 60°C) and mounted in Prolong Gold (Invitrogen) with 1.5 μg/ml DAPI. FITC-labeled chromosome 6 paint (Cambio) was prepared in 7.5 μl hybridization buffer. *Cd4* and *Cd8* probes were resuspended in 7.5 μl hybridization buffer. Probes were mixed just prior to hybridization.

Immunofluorescence

Cells were adhered to poly-L-lysine-coated coverslips, fixed (2% paraformaldehyde/PBS, 10 min), permeabilized (0.4% Triton/PBS, 5 min), and blocked (2.5% BSA/0.1% Tween/10% goat serum/PBS, 30 min). RUNX3 detection used a rabbit RUNX3 antibody (Egawa et al., 2007) (1:50,000 in blocking solution, 1 hr). Cells were rinsed (0.2% BSA/0.1% Tween-20/PBS) and incubated in goat anti-rabbit Alexa 488 (Invitrogen, 1:500 in blocking solution, 1 hr). Coverslips were rinsed (0.1% Tween-20/PBS) and mounted in Prolong Gold with DAPI.

Microscopy and Analysis

Optical sections of 80 nm *x-y* pixel size and separated by 0.3 μm were acquired by confocal laser scanning microscopy (Leica SP5, 100×/1.4 oil objective). Only cells with signals from both alleles (typically >95%) were analyzed with

Leica software. At least three independent experiments were performed (n = 166 to 356 alleles for *Cd4-Cd8* association, see Supplemental Tables for one representative experiment of PCH analysis). Distances between the center of the *Cd4* and *Cd8* signals was measured with Image J software.

The empirical interallelic distance distributions were compared to test whether they had been drawn from the same underlying continuous distribution. The statistical significance of pair-wise distributions' dissimilarity was assessed with the nonparametric two-sample Kolmogorov-Smirnov (KS) test (Massey, 1951). The reported p values were calculated with MATLAB 7.9 (The MathWorks Inc., Natick, MA).

Association of *Cd4* and *Cd8* with pericentromeric domains was scored if the loci signals were juxtaposed or overlapping with γ-satellite signals. Statistical significances for PCH localization were calculated with χ^2 test (Campbell, 1989). Yates' correction was applied when any category had less than 10 observations. Each data set was paired with the most relevant stage, genotype, or cell type.

RT-PCR

RNA was extracted with TRIZOL (Invitrogen). Reverse transcription was performed with Superscript III (Invitrogen), cDNA analyzed in triplicate with Quantitect Multiplex PCR Mix (QIAGEN) for Taqman probes or iQ SYBR Green Supermix (BioRad) in the iCycler (BioRad), and normalized to *beta-actin* (*Actb*). Primer sequences: *Actb*: 5′-GCTCTGGCTCCTAGCACCAT, 3′-GCCAC CGATCCACACAGAGT, probe: FAM-TCAAGATCATTGCTCCTCCTGAGCGC-TAMRA; *Cd4*: 5′-GACTGACCCTGAAGCAGGAG, 3′-CTGTCTGGTTCACCC CTCTG; *Cd8a*: 5′-CACAGGAGCCGAAAGCGT, 3′-GGGCTTGCCTTCCTGT CTG. Standard error bars were calculated from two to four independent experiments.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and five tables and can be found with this article online at doi:10.1016/j.immuni.2011.03.004.

ACKNOWLEDGMENTS

We would like to thank J. de Nooij and T. Jessell for RUNX3 antibody (Kramer et al., 2006). We also thank members of the J.A.S. and D.R.L. labs for thoughtful discussions and critical comments on the manuscript. This work was supported by a Leukemia and Lymphoma Scholar Award, an NIH 1R01GM086852 grant, and a WT project grant (WT 085096) (J.A.S.). A.C. and D.R.L. were supported by funds from the Howard Hughes Medical Institute and from the Helen and Martin Kimmel Center for Biology and Medicine. S.L.H. is supported by a Fellow Scholar Award from the American Society of Hematology and M.M. is supported by NSF IGERT grant 0333389. M.M.W.C. was funded sequentially by a Postdoctoral Fellowship from the Cancer Research Institute and a Senior Fellowship from the Helen and Martin Kimmel Center for Stem Cell Biology. A.E.C. and D.J.B. are supported by a BBSRC project grant (Biotechnology and Biological Sciences Research Council). Work in the lab of W.E. is supported by the Austrian Science Fund (FWF; P19930). Work in the lab of R.B. is supported by the Intramural Research Program of the National Cancer Institute, Center for Cancer Research, NIH. M.S. is an Irvington Institute Fellow of the Cancer Research Institute.

Received: July 1, 2010
Revised: September 7, 2010
Accepted: December 21, 2010
Published online: March 24, 2011

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