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*J Immunol* published online 31 August 2011
http://www.jimmunol.org/content/early/2011/08/29/jimmunol.1100709

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2011/08/29/jimmunol.1100709.DC1

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Modulation of the Murine CD8 Gene Complex Following the Targeted Integration of Human CD2-Locus Control Region Sequences

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The Journal of Immunology, 2011, 187: 000–000.

locus control regions (LCRs) are cis-acting gene-regulatory elements functionally defined by their ability to direct expression of a linked transgene in a position-independent, copy number-dependent, and tissue-specific manner (1). The human CD2 (hCD2)-LCR directs T cell-specific expression of linked transgenes and has been used to generate a number of useful transgenic mouse lines (2, 3). Within the hCD2 gene locus, the LCR is located in the 3′ flanking region of the hCD2 gene and is characterized by three DNaseI hypersensitive sites (HS1–3) (4, 5). Whereas HS1 of the hCD2-LCR was shown to contain classic enhancer activity in transient-transfection assays, HS3 was shown to be essential for the protection from position effects in transgenics (4–6). Transgenic mice carrying the hCD2 minigene linked to the full LCR achieved position-independent expression in all thymocytes and T cells (4). In contrast, transgenic mouse lines that lack HS3 of the LCR exhibited variagated CD2 expression when the transgene integrated in centromeric positions (4). However, these experiments could not answer the question whether HS3 by itself is sufficient to remodel a lymphoid locus and force its expression in all lymphoid cells, as the full hCD2-LCR does.

Random integration and frequent multiple copy integration of transgenes are two aspects of transgenesis that are difficult to control and, as a consequence, complicate direct comparison of transgenic mouse lines. Fluorescence in situ hybridization to identify the chromosome composition and chromosomal position of the transgene-integration site has been used to address this problem. Other approaches take advantage of the Cre/lox site-specific recombination systems to reduce transgene copy numbers, as well as to allow direct comparison of transgenic lines with the same integration site but different copy numbers (7, 8). This system also allows the generation of conditionally targeted mouse lines in which deletion of gene sequences is controlled in a cell lineage-specific and developmental stage-specific manner through the expression of Cre recombinase.

Three distinct developmental stages of T cell differentiation in the thymus can be defined based on the expression pattern of the coreceptors CD4 and CD8. Immature thymocytes are negative for the expression of both coreceptors and are known as double-negative (DN) thymocytes. Commitment to the αβ TCR T cell lineage prompts these immature thymocytes to proceed to the double-positive (DP) stage, simultaneously expressing both CD4 and CD8 coreceptors. Subsequent commitment to either the CD4 single-positive (SP) or CD8 SP cell fate results in two mature T cell lineages in which the expression of the CD4 and CD8 coreceptors is mutually exclusive. Several cis-regulatory elements responsible for the differential turning on and off of these genes during thymocyte development have been identified. For the CD4 coreceptor, regulatory elements have been identified that are involved in both positive and negative regulation of CD4 gene expression (reviewed in Ref. 9).

The CD8 coreceptor is expressed as CD8α and CD8β heterodimers in most thymus-derived T cells. However, some cells, such as intraepithelial lymphocytes from the gut or CD8+ dendritic cells, express only CD8α homodimers (10–12). The two CD8 chains are encoded by two distantly related, but closely linked,
Expression patterns suggest that the CD8 lineage-specific expression of the CD8 receptor (13–20). Their remnants, are required for the appropriate developmental stage- and condition-dependent expression of the CD8 coreceptor. Removal of HS1,2 of the hCD2-LCR in the mCD8 gene complex. We report that the continuous presence of the hCD2-LCR is required for its effect on the expression of the CD8 genes and that cells, influenced by the LCR during development, can convert to their destined phenotype once the LCR is removed. Insertion of HS1.2 of the hCD2-LCR in the mCD8 gene locus have similar effects as the full LCR. In contrast, knock-in mice with only HS3 of the LCR inserted display no overt effect on the expression pattern of the CD8 coreceptor.

Material and Methods

**Generation of mCD8–(hCD2-LCR)–targeting constructs (CD8/LCR1, 12, CD8/LCR3, CD8/xLCRix)**

To generate the targeting constructs for insertion of Dnasel HS1.2 or HS3 of the hCD2-LCR, a similar approach was used to that previously described (22). A 1.3-kb HindIII-Xmal fragment (HS1.2) or a 0.8-kb Xmal-HindIII fragment (HS3) of the LCR was ligated into a HindIII/EcoRV linearized pBlueScript SK plasmid containing the neomycin gene flanked with loxP sites (23). The fragments containing 1x-neo-lox and hCD2-LCR sequences were lifted from their vector by digestion with SalI-NotI, blunt-ended, and ligated to the HpaI site of a subcloned 6.3-kb BamHI fragment of the mCD8 gene complex. This genomic CD8 fragment in pBlueScript SK contains Dnasel HS1 of cluster III and downstream sequences of the CD8 gene complex and provided a 3.9-kb 5’ homology and a 2.4-kb 3’ homology for the respective targeting constructs. A 2.7-kb BamHI/HindIII fragment containing the HSV-TK gene for negative selection was ligated from the pPNT vector (24), blunt-ended, and ligated into the Smal site of each particular targeting vector. Both constructs were linearized with NotI and used for embryonic stem (ES) cell transfection.

To insert the hCD2-LCR flanked by loxP sites into the mCD8 gene complex, a loxP site was ligated to the 3’ HindIII site of the hCD2-LCR in a pBlueScript SK plasmid. The hCD2-LCR-loxP fragment was lifted from its vector and inserted 3’ of the neomycin gene flanked with loxP sites contained in a pBlueScript SK plasmid (23). The fragment containing sequences of 1x-neo-lox-(hCD2-LCR)-loxP was lifted from its vector and inserted into the HpaI site of a subcloned 6.3-kb BamHI fragment of the mCD8 gene complex. For negative selection, the HSV-TK gene was lifted from the pPNT vector (24) and ligated into the Smal site of the targeting vector. The vector was linearized by NotI digestion before being used for ES cell transfection.

**Transfection and selection of ES cells**

ES cells (recombinase+ PC3 cells) were maintained in their undifferentiated state by growth on a feeder layer of gamma-irradiated primary embryonic fibroblasts, as described previously (25). Electroporation of 0.8 × 10^6 ES cells with 50 mg/ml linearized targeting constructs and selection of homologous recombinated clones with G418 (300 mg/ml) and ganciclovir (2 mM) were carried out as previously described (24). Neoc expression pattern of the CD8 receptors was confirmed by Southern blot analysis for line-specific detection of the floxed neomycin gene, leaving behind a single loxP site next to either 1.3- or 0.8-kb hCD2-LCR sequences, respectively, in the CD8 gene complex. The Cre recombinase transgene, derived from the PC3 cell background, was bred out in the process of backcrossing. Comparison of mice, derived from the two independent ES clones for each CD8/LCR-targeting event, revealed similar phenotypes; therefore, backcrosses to C57BL/6 and homozygous CD8/LCR knock-in mouse lines were generated from one clone only for each line. All mice used for these studies descend from this backcross for a minimum of 10 times to C57BL/6 mice. Data described below result from knock-in mice homozygous for the integration of the full or partial hCD2-LCR or from knock-in mice heterozygous for the integration of the floxed hCD2-LCR and positive or negative for a developmental stage-specific Cre transgene.

**Southern blot analysis**

Genomic DNA was extracted from sorted ES cell clones, tail biopsies, T cell-enriched spleen cells, or total thymus cells by phenol/chloroform extraction and ethanol precipitation. DNA samples were digested overnight with the recommended concentration of the restriction enzyme EcoRv (Roche) and resolved on 1% agarose gels. Southern blots were set up to immobilize the samples on Hybond XL nylon membranes (GE Healthcare) and hybridized with probes labeled with [ 32P]dCTP (GE Healthcare). DNA probes specific for the CD8 locus were used to identify the targeted and wild-type or deleted CD8 allele (22).

**Flow cytometric analysis**

For sample analysis by flow cytometry, single-cell suspensions from spleen, lymph node, or thymus were prepared, and 10^6 cells were stained with the following Abs in appropriate combinations: (FITC) anti-TCRβ (H57-597), (PE) anti-CD4 (RM 4-5), (PerCP) anti-CD8α/β-Ly-2 (53-6.7; BD Pharmingen), (allophycocyanin) anti-CD8α (53-6.7), (allophycocyanin) anti-CD8β/ Ly-3 (eBioscience), and (allophycocyanin) anti-CD3 (H57-597). Three- and four-color FACSCalibur flow cytometry was carried out on a FACSCalibur machine, and data were analyzed using FlowJo software. Abs were obtained from eBioscience, unless stated otherwise. To identify immature thymocytes, total thymocytes from three or four thymi were depleted by incubation with a mixture of biotinylated Abs against the following Abs: anti-TCRβ, anti-CD4, anti-CD19, anti-CD11b, anti-NK1.1, and anti-Ter119, followed by incubation with Streptavidin beads (Miltenyi Biotec). Cells were separated using an autoMACS cell separator. Negatively selected cells were stained using (PE) anti-CD3, (FITC) anti-CD44, (allophycocyanin) anti-CD25, and (PerCP) anti-CD8α or (PerCP) anti-CD8β Abs, to identify DN subpopulations.

To identify γδ TCR+ cells, negatively selected cells from individual organs were stained using (PE) anti-TCRγδ, (FITC) anti-CD4, anti-CD19, anti-CD11b, anti-NK1.1, and anti-Ter119, followed by incubation with Streptavidin beads (Miltenyi Biotec). Cells were separated using an autoMACS cell separator. Negatively selected cells were stained using (PE) anti-CD3, (FITC) anti-CD44, (allophycocyanin) anti-CD25, and (PerCP) anti-CD8α or (PerCP) anti-CD8β Abs, to identify DN subpopulations.

For lineage identification of peripheral cells or bone marrow cells and CD8 expression, the following Abs were used: (allophycocyanin) anti-CD19, (FITC) anti-IP30, (PE) anti-CD8a, (allophycocyanin) anti-CD8β, Abs (FITC) anti-CD44, (FITC) anti-TCRβ, and (FITC) anti-B220 were included to allow exclusion of these cells by gating.

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**Cell sorting**

For the isolation of distinct T cell populations, single-cell suspensions were prepared from spleen and lymph node pooled from up to 20 mice from line CD8/LCR (cII-III). Cells were stained for anti-TCRβ, CD4, CD8, and CD44 and sorted using a MoFlo cell sorter (Beckman Coulter).

**Adaptive cell transfer**

Rag2^−/− recipient mice were injected i.v. with either 0.8 × 10^6 sorted CD4hi (CD8hi) peripheral T cells from either line CD8/LCR (cII-III) or line
CD8/LCR 1.2. Donor-derived lymphocytes in host mice were analyzed by flow cytometry a number of weeks after cell transfer.

**Results**

**Generation of floxed mCD8–(hCD2-LCR) knock-in mice**

It was postulated that LCRs effect their action either by setting up active chromatin structures, but may not be required subsequently, or by obligatory continuous interactions with the regulatory elements and promoters in their proximity. To distinguish between these two possibilities, we targeted the hCD2-LCR flanked by loxP sites into the intergenic region of the endogenous mouse CD8 gene complex between DNaseI hypersensitive clusters II and III. The generated knock-in mouse line was designated CD8/lxLCRlx (cII-III) (Fig. 1A). Deletion of the inserted LCR at specific stages during hematopoietic development was achieved by crossing CD8/lxLCRlx (cII-III) knock-in mice to transgenic mice with lineage- and stage-specific expression of Cre (2, 26).

**Developmental stage-specific deletion of the inserted hCD2-LCR by Cre recombination**

To see the effect of deletion in hematopoietic stem cells, knock-in mice were crossed to vav-iCre transgenic mice (2). For LCR deletion in early thymocytes, knock-in mice were crossed to hCD2-iCre transgenic mice; for deletion in mature T cells, they were crossed to dLck-iCre transgenic mice (2, 26).

Resulting mice, positive or negative for the Cre recombinase transgene and heterozygous for the insertion of the hCD2-LCR, were analyzed by FACS (Fig. 1B–E, Supplemental Fig. 1). CD8/lxLCRlx knock-in mice negative for a Cre transgene deregulate the

**FIGURE 1.**  CD8/lxLCRlx (cII-III) knock-in mice and CD8 coreceptor expression. A, The targeted CD8 gene complex showing the integration site of the floxed hCD2-LCR in relation to the CD8α and CD8β genes. B, BamHI; H, Hpa I; dotted box, hCD2-LCR; triangular boxes, loxP sites; filled boxes, translated exons; open box, untranslated exon. Locations of DNaseI-HS clusters are shown by vertical arrows. Restriction enzyme sites are indicated by vertical bars. Horizontal lines indicate the relative distance of the hCD2-LCR integration site to the promoters of the CD8 genes. B–E, Deletion of the inserted hCD2-LCR by developmental stage-specific Cre expression. CD8 coreceptor expression on αβ TCR+ spleen cells from CD8/lxLCRlx (cII-III) knock-in mice negative or positive for a CRE transgene analyzed by plotting CD4 against CD8α or CD8β, respectively. B, vav-iCre. C, hCD2-iCre. D, dLck-iCre. E, CD8 coreceptor profile on CD4+ T cells from knock-in mice crossed to dLck-iCre transgenics.

**FIGURE 2.** Late thymocyte-specific deletion of the inserted hCD2-LCR by dLck-iCre expression. Upper panel, Southern blot analysis of sorted peripheral T cells of dLck-iCre+ CD8/lxLCRlx knock-in mice. The band for the wild-type or deleted allele (13 kb) and the band for the knock-in floxed allele (4.4 kb) are indicated by arrows. Lower panel, FACS blot shows CD4/CD8 expression profile of cell populations used for DNA analysis.
expression of the CD8 coreceptor, as seen previously in knock-in mice with constitutive insertion of the hCD2-LCR (Fig. 1B–E) (22).

Deletion of the floxed hCD2-LCR in hematopoietic stem cells or immature lymphocytes by expression of a var-iCre or a hCD2-iCre transgene, respectively, results in mice in which the developmental-expression pattern of the CD8 genes is not perturbed compared with wild-type. This indicates that the specific subsequent expression pattern of the CD8 coreceptor is not affected by the presence of the hCD2-LCR during embryonic development (Fig. 1B, Supplemental Fig. 1A) or at early stages during thymocyte development (Fig. 1C, Supplemental Fig. 1B).

In CD8/lxLCRlx mice positive for the transgene dLck-iCre, the deletion of the LCR is effected at a late (post-DP) stage of lymphocyte development (26). In such mice, the LCR establishes an open chromatin configuration in DN3-DN4 and CD4⁺ thymocytes, and these cells are positive for CD8 expression. However, upon deletion of the LCR after the positive-selection stage, a large number of the CD4⁺(CD8⁺) mature thymocytes convert to a CD4⁺CD8⁻ phenotype (Supplemental Fig. 1C). In dLck-iCre⁺ knock-in mice, 38% of all TCRhigh thymocytes are CD4 SP compared with 62% in wild-type mice (Supplemental Fig. 1C).

Peripheral CD4⁺ T cells from spleen of dLck-iCre⁺ knock-in mice exhibit an intermediary phenotype for CD8 coreceptor expression between Cre negative knock-in mice and wild-type mice, which indicates a gradual ceasing of CD8 coreceptor expression in CD4⁺ cells (Fig. 1D, 1E). In Cre⁺ CD4⁺ splenocytes 18% of cells express the CD8 coreceptor as CD8αβ heterodimers compared with 77% in Cre⁻ knock-in mice (Fig. 1E). This indicates that cells that have been affected by the hCD2-LCR can convert to their destined phenotype once the LCR is removed through Cre-mediated recombination.

FIGURE 3. Age-related influence on CD8 coreceptor expression in CD8/lxLCRlx knock-in mice negative or positive for dLck-iCre transgene. A. Comparison of CD8 coreceptor expression in CD4⁺ T cells between young and old CD8/lxLCRlx mice negative or positive for the dLck-iCre transgene. Percentages of CD4⁺ CD8⁻ and CD4⁺(CD8⁺) cells are expressed in relation to total CD4⁺ T cells. The age of mice is shown in months. B. Percentages show average value of CD4⁺(CD8⁺) cells at different ages in relation to mice aged 1.5 mo. Error bars show SD.
Southern blot analysis of CD8/lxLCRlx knock-in mice

To assess the deletion of the inserted hCD2-LCR through Cre-mediated recombination at the genomic level, Southern blot analysis was performed on CD4+ cells sorted in CD4+ CD8-, CD4+CD8med, and CD4+CD8β subpopulations (Fig. 2).

The high expression level for the CD8 co-receptor in CD4+CD8β T cells is reflected in the intense band for the targeted knock-in allele. In CD4+CD8med T cells, the lower level of CD8 co-receptor expression is associated with a faint band for the floxed knock-in allele. This indicates that within the CD4+CD8med population, some cells have not deleted the inserted LCR. However, a proportion of these cells seem to have lost the LCR but continue to express the CD8 co-receptor at low levels on the surface, suggesting a possible delay between deletion of the LCR and extinction of CD8 cell surface expression. In CD4+CD8β cells, the absence of a band for the targeted knock-in allele corresponds to the termination of CD8 co-receptor expression in these cells (Fig. 2). The deletion of the LCR appears complete in CD8+ SP cells, since no band for the floxed knock-in allele is detectable, indicating that these cells convert to wild-type expression levels for the CD8 co-receptor, as shown by mean fluorescence intensities (Supplemental Fig. 2A).

To confirm tissue and developmental stage-specific deletion of the hCD2-LCR by the activity of the Cre recombinase, total thymocytes, T cell-enriched spleen cells, or tail tissue from CD8/lxLCRlx knock-in mice negative or positive for the lxLCRlx knock-in mice transgene were analyzed (Supplemental Fig. 2B). Southern blot analysis of total thymocytes (~90% DP cells) from dLck-iCre+ knock-in mice confirmed that the hCD2-LCR is still present in immature thymocytes, indicating that the Cre recombinase has not yet been active. A weak band for the targeted allele in T cell-enriched spleen cells and the strong band in tail tissue from dLck-iCre+ knock-in mice prove the specificity of the Cre recombinase activity.

**Influence of age on CD8 expression on CD4 cells in CD8/-lxLCRlx knock-in mice**

FACS analysis of CD8/lxLCRlx mice positive for the dLck-iCre transgene identified a proportion of CD4+ cells, which maintain expression of the CD8 co-receptor, despite migration to peripheral lymphoid organs (Fig. 1D, 1E). Southern blot analysis of these CD4+CD8β cells confirmed that these cells have not deleted the inserted hCD2-LCR (Fig. 2). To assess whether the complete deletion of the LCR and the loss of CD8 expression in the CD4 lineage are a function of time, CD4 and CD8 co-receptor expression profiles of young and old mice were compared. Such analysis revealed a decrease in the percentage of peripheral CD4+CD8β cells with increasing age. Whereas in young (1.5-mo-old) knock-in mice, ~17% of all CD4+ cells express the CD8 co-receptor, in old (12-mo-old) knock-in mice the proportion decreases to ~6% (Fig. 3). The persistence of CD4+CD8β cells throughout the lifespan of dLck-iCre+ CD8/lxLCRlx knock-in mice indicates that the deletion of the inserted hCD2-LCR by Cre recombinase is not complete. This is consistent with reports that recombination driven by the dLck-iCre transgene is less efficient within CD4+ lymphocytes than in CD8+ lymphocytes (26). However, peripheral CD4+CD8β cells could represent recent emigrants from the thymus, in which deletion of the inserted LCR by Cre activity is delayed. The continuous presence, but decreasing number, of CD4+CD8β cells with age could be attributed to the reduced thymic output due to involution of the thymus. In CD8/LCR (cII-III) knock-in mice with constitutive insertion of the hCD2-LCR, a reduction in CD4+CD8β cells with age has been detected. Characterization of those mice indicated a link between the attenuated influence of the hCD2-LCR on CD8 coreceptor and the proliferation history of a cell (22). However, in dLck-iCre+ CD8/lxLCRlx knock-in mice, the decrease in CD4+CD8β cells is more pronounced as a result of the deletion of the LCR (Fig. 3B).

**Generation of CD8/LCR knock-in mouse lines with partial LCR sequences**

To identify the ability of each of the DNaseI hypersensitive regions within the hCD2-LCR to overcome heterochromatinization, constructs containing either hypersensitive regions HS1,2 or only hypersensitive region HS3 of the hCD2-LCR were inserted in the mCD8 locus, and the resultant mice were compared with mice containing the full LCR (Fig. 4) (22). The knock-in lines generated were designated CD8/LCR1,2 and CD8/LCR3, respectively.

**Expression of CD8 genes on cells from CD8/LCR knock-in mouse lines**

Insertion of HS1,2 of the hCD2-LCR results in the expression of CD8αβ and CD8β in CD4+ cells, similar to mice with a full LCR knocked in the CD8 locus (Fig. 5A, 5B) (22). In both knock-in lines, ≥75% of CD4+ cells express the CD8 co-receptor as CD8β heterodimers, whereas ≥9% of CD4+ cells express the CD8 co-receptor as CD8αβ homodimers (Fig. 5C).

Interestingly, in both of these knock-in lines, some lymphocytes are CD4 SP, indicating a resistance to the activating effect of the LCR sequences on the CD8 gene complex. These CD4 SP cells seem to follow their normal developmental program. HS1,2 of the hCD2-LCR, which can be subject to position effects in transgenesis (4), seem sufficient to deregulate the CD8 gene complex in the majority of cells within a lineage, which is normally programmed to turn off CD8 expression.

In contrast, HS3 sequences of the hCD2-LCR seem insufficient for overcoming the endogenous regulation of the CD8 gene.
complex, because no deregulation of CD8 coreceptor expression was observed in peripheral lymphocytes from CD8/LCR3 knock-in mice. This suggests that HS3 of the LCR, which is essential for the protection of position effects in transgenic mice (4), is unable, by itself, to influence the CD8 gene complex.

Enhanced expression of the CD8α and CD8β-chain, as measured by mean fluorescence intensity, was observed in CD8 SP cells in peripheral lymphocytes from knock-in lines CD8/LCR (cII-III) or CD8/LCR1,2, but not from knock-in line CD8/LCR3. No change in ratio between CD4+ cells and CD8 SP cells was observed, indicating that the insertion of hCD2-LCR sequences does not influence the cell lineage decision of immature lymphocytes. Mature thymocytes, identified by high αβTCR expression, from each CD8/LCR knock-in line show a similar CD8 expression profile as observed in peripheral T cells (data not shown).

Expression of transgenes under the regulation of the hCD2-LCR was shown from the early stages of thymocyte development onward (2). By the DN3 stage, almost all cells from knock-in lines CD8/LCR (cII-III) and CD8/LCR1,2 express the CD8α-chain. However, in cells from knock-in line CD8/LCR3 no expression of the CD8 coreceptor is detected. In thymocytes at the DN4 stage almost 100% from knock-in lines CD8/LCR (cII-III) and CD8/LCR1,2 express the CD8α coreceptor (Fig. 6A) (mainly as CD8αα homodimers; data not shown). CD8 expression detected in thymocytes from wild-type and CD8/LCR3 at the DN4 stage is attributed to immature CD8 SP cells most likely included in the cell preparation due to the staining protocol. This analysis of immature thymocytes revealed that HS3 of the LCR is sufficient to override the regulation of the CD8 coreceptor during early T cell development. However, HS3 of the LCR alone is unable to initiate expression of the CD8 coreceptor (Fig. 6A).

To assess how T cells, which do not normally open the chromatin of the CD8 gene complex during their development, are affected by the inserted hCD2-LCR sequences, γδTCR+ T cells from thymus and spleen of the CD8/LCR knock-in mice were analyzed (Fig. 6B, Supplemental Fig. 3A).

In two knock-in lines, lines CD8/LCR (cII-III) and CD8/LCR1,2, a large proportion of γδTCR+ cells from thymus (≥91%) and spleen (≥74%) express the CD8 coreceptor either as CD8αα homodimers or CD8αβ heterodimers. Interestingly, both knock-in lines have a significant population of γδTCR+ CD8− cells in thymus and spleen, suggesting that some cells are able to elude the influence of the inserted hCD2-LCR sequences (Supplemental Fig. 3A). In contrast, γδTCR+ cells from knock-in line CD8/LCR3 show no increase in CD8 coreceptor-expressing cells containing only a small population of γδTCR+ CD8+ cells in thymus and spleen, similar to the ones observed in wild-type mice. These data suggested the HS3 sequences of the hCD2-LCR are unable to initiate CD8 coreceptor expression in cells, which normally do not express CD8. However, HS1,2 sequences of the LCR or the complete hCD2-LCR can initiate expression of the CD8 coreceptor in a large proportion of γδTCR T cells, which are not programmed to express the CD8 genes.

Analysis of non-CD8 coreceptor-expressing lymphoid and myeloid cell lineages by flow cytometry revealed the inserted hCD2-LCR sequences cannot activate CD8 expression in these lineages (data not shown). This indicated that the effect of the LCR is probably restricted to T lineage lymphoid cells.

Age-related loss of hCD2-LCR–dependent CD8 expression on CD4 cells

To determine whether individual HS regions of the hCD2-LCR are responsible for the loss of LCR influence on the CD8 gene complex,
cohorts of young and old knock-in mice from line CD8/LCR1,2 and CD8/LCR3 were compared with CD8/LCR (cII-III) mice (Supplemental Fig. 3B). These experiments showed a similar increase of CD4+ CD8- lymphocytes with age, as previously shown for mice with the full hCD2-LCR inserted in the CD8 locus (22).

Knock-in mice with only HS3 sequences of the LCR inserted (CD8/LCR3) show no change in their CD4 and CD8 expression profile with age. We conclude from this analysis that the information contained within the HS1,2 sequences of the LCR is sufficient to exert the full effect of the hCD2-LCR on CD8 coreceptor expression.

To follow the loss of CD8 expression on CD4 cells more precisely, we sorted peripheral CD4+(CD8+) T cells from either knock-in line CD8/LCR (cII-III) or CD8/LCR1,2 and transferred them into Rag2-/- mice (Supplemental Fig. 4). The transferred cells are induced into rapid proliferative expansion within the lymphopenic environment of the host. As described previously, a loss of CD8 expression was observed in donor-derived CD4+ T cells (22); however, no significant difference between the knock-in lines with full or HS1,2 hCD2-LCR sequences was detected. This suggests that, when inserted within the CD8 gene complex, HS1,2 sequences implement a similar effect to the full length hCD2-LCR (Supplemental Fig. 4).

Discussion

Lineage- and developmental stage-specific deletion of the inserted hCD2-LCR

Targeted insertion of an LCR as a single entity into a defined genomic region has been shown to influence the expression of endogenous genes (22, 27). Using a Cre recombinase-based approach, we addressed the question of whether the continuous presence of an LCR is required to exert this effect.

No effect on the CD8 coreceptor-expression profile was observed in CD8/ixLCRlx knock-in mice when the LCR was deleted during early development by the expression of a vav-iCre or hCD2-iCre transgene, respectively (Fig. 1B, 1C). This suggests that the temporary presence of the LCR during early hematopoietic development (stem cell stage and early lymphoid development, respectively) does not influence the subsequent developmental stage-specific expression pattern of the CD8 gene.

Cell lineage-specific deletion of the inserted hCD2-LCR at a later lymphoid-specific stage, after the effects of the LCR have been manifested, causes a conversion of the deregulated CD8 coreceptor expression to its wild-type expression pattern. The expression of the CD8 coreceptor in CD4+ cells was shown to be directly linked to the presence of the hCD2-LCR (Fig. 2). Taken together, these data indicated that the inserted hCD2-LCR does not “indelibly mark” the CD8 gene complex and that the continuous presence of the LCR is required for the deregulation of the normal pattern of CD8 expression. Cells whose expression of the CD8 coreceptor is influenced by the inserted hCD2-LCR can convert to their destined phenotype after deletion of the LCR.

A declining influence of the LCR on the expression of the CD8 coreceptor, which correlates with the proliferation history of a cell, was reported for CD8/LCR knock-in mice with a constitutively inserted LCR (22). Such a loss in LCR activity was also observed in CD8/ixLCRlx+/cre- knock-in mice. Although it cannot be excluded that some CD4+ CD8- lymphocytes from CD8/ixLCRlx+/ 

dLck cre knock-in mice no longer express the CD8 coreceptor due to such an inactivation of the LCR, the majority of cells lose CD8 coreceptor expression as a result of LCR deletion (Fig. 3).

Targeted deletion of the endogenous mouse β-globin LCR to address the role of the LCR in its native locus revealed a contributory, rather than exclusive, role in transcriptional regulation. The dominant role of the β-globin LCR was described as conferring high-level transcription of the globin genes, whereas its role in establishing and maintaining an open chromatin configuration was dispensable (28). In contrast, in γβ-thalassemia patients, the deletion of the β-globin LCR resulted in an inactive chromatin configuration of the β-globin genes in vivo, which prevented expression of the globin genes (29). In the context of ectopic integration of a single LCR, the continuous presence of the LCR seems essential for the influence on endogenous genes, as shown in the generated CD8/ixLCRlx knock-in mice.

When used to drive expression of a linked transgene, the LCR acts in concert with the promoter of the transgene to achieve tissue-specific and position-independent expression (30). However, expression of endogenous genes adjacent to the integration site of a transgenic construct can also be influenced by the inserted LCR. In CD8/LCR knock-in mice, the inserted hCD2-LCR interacts with the promoters of the endogenous CD8 genes and influences the developmentally regulated expression of these genes. As a consequence of the presence of the LCR, some cell lineages (e.g., CD4+ cells) that are normally programmed to silence the CD8 genes at a certain stage of development are prevented from doing so. However, as shown previously, the endogenous CD8 gene-repressive regulatory processes in these cells appear to continuously contest the influence of the inserted LCR, which can result in the eventual inactivation of the LCR (Fig. 3) (22). Therefore, deletion of the inserted LCR by Cre-mediated recombination facilitates the conversion of these cells to their destined phenotype. This apparent reversibility of the LCR influence indicates that the endogenous CD8 genes are not permanently marked by the temporary presence of the LCR and that the continuous presence of the LCR is essential for the deregulation of the endogenous CD8 genes.
Influence of partial hCD2-LCR sequences on the mCD8 gene complex

In addition, to further functionally characterize the specific regions of the hCD2-LCR, we generated knock-in mice with partial LCR sequences inserted in the mCD8 locus. The three defined DNaseI HSs (HS1–3) that make up the hCD2-LCR have been extensively characterized in transgenic mice (4, 6). The generation of CD8/LCR knock-in mice with partial hCD2-LCR sequences allowed further characterization of the DNaseI HSs of the LCR when inserted in a defined locus, making possible the direct comparison of their influence on the endogenous CD8 genes, unlike in transgenic mouse models where random construct integration often obfuscates analysis (4, 8).

Whereas lineage-specific and position-independent transgene expression requires the full LCR (4), HS1,2 of the hCD2-LCR seem to be sufficient for this effect in CD8/LCR knock-in mice. In both knock-in lines, CD8/LCR (cII-III) and CD8/LCR 1,2, peripheral CD4+ cells are prevented from closing the CD8 locus, expression of the CD8 coreceptor is initiated in immature thymocytes, and the majority of γδTCR+ cells are forced to express the CD8 coreceptor (Figs. 5, 6, Supplemental Fig. 3A). Furthermore, in CD8/LCR 1,2 mice, a similar resistance to the inserted LCR sequences was observed as in CD8/LCR (cII-III) mice, which is shown in the decline of CD4+(CD8+) cells with age and after adoptive transfer (Supplemental Figs. 3, 4). Interestingly, the insertion of HS3 alone of the LCR does not result in a changed expression of the endogenous CD8 genes, indicating that, as an individual region, HS3 is unable to influence the regulation of adjacent genes under the circumstances described in this article. It is possible that heterochromatinization at centro- or telomeric sites is different from the one occurring in T cells that close-down the CD8 gene complex. Whereas HS3 is essential to avoid the former type of heterochromatinization, it may be dispensable in a more favorable environment, such as the nucleus of T lymphocytes.

The analysis of CD8/LCR knock-in mice complements the characterization of LCRs using transient and transgenic assays. It was reported that LCRs contain a core sequence with enhancer activity in transient assays but that does not function as an LCR in transgenic assays. However, when additional flanking elements, without measurable activity on their own, are included with the core sequence, LCR activity is observed (reviewed in Ref. 31).

For the hCD2-LCR, HS1,2 contain the core sequence with enhancer function, and HS3 is the flanking sequence, which is required for complete LCR function in transgenic mice. In CD8/LCR knock-in mice, the core sequence of the hCD2-LCR influences the regulation of the CD8 genes in the same manner as does the full-length LCR, indicating that inclusion of HS3 has no additional/synergistic effect. This suggests that, when inserted as an isolated unit, the core sequence of the LCR contains all functional elements needed to influence the regulation of the endogenous CD8 genes. However, to confirm this suggestion, other endogenous gene loci adjacent to inserted LCR sequences need to be analyzed.

In the described CD8/LCR knock-in mice, interaction of the inserted LCR sequences with the adjacent gene-regulatory regions of the endogenous CD8 genes was assessed by examining the expression pattern of the CD8 coreceptor. The activity of the hCD2-LCR coincides with the establishment of hypersensitivity and accessibility of the endogenous CD8 locus before the onset of CD8 transcription (2, 32). Therefore, the initiation of CD8 coreceptor expression in immature thymocytes at the DN3 stage in CD8/LCR knock-in mice coincides with this preparative stage of the chromatin environment of the CD8 gene locus. Thus, the LCR initiates expression in DN thymocytes at the same time that the CD8 locus is being prepared for normal CD8 expression (32). This preparative stage of the CD8 locus chromatin region may explain the readiness of immature thymocytes to express the CD8 receptor in the presence of specific LCR sequences (Fig. 6A). Chromatin accessibility may also explain why the partial LCR sequences HS1,2 effect the same phenotype as does the full-length LCR.

Bivalent chromatin as the basic mechanism that influences gene regulation at key points in thymocyte differentiation and lineage-fate decisions has been suggested by Harker et al. (32) and others (33–35). Remodeling of the CD8 promoter was reported at the DN3 stage (32), and this coincides with the completion of lineage divergence of the γδ and γδ T cell lineages (reviewed in Ref. 36). This overlap of lineage-commitment decision and poised chromatin accessibility of the CD8 gene complex may explain the increase in CD8 coreceptor-expressing γδ TCR+ thymocytes observed in knock-in lines CD8/LCR (cII-III) and CD8/LCR,1,2 as opposed to what occurs in wild-type mice (37, 38). Interestingly, expression of the CD8 coreceptor in γδ TCR+ thymocytes from CD8/LCR knock-in mice can occur in the CD8αβ heterodimer or CD8αα homodimer form, which is also observed in the peripheral γδ T cell lineages of these mice. Some cell lineages (e.g., γδ TCR intraepithelial lymphocytes and dendritic cells) express only the CD8α gene; it will be interesting to study the influence of the hCD2-LCR sequences on the expression of the CD8β gene in these cells.

The absence of a measurable influence of the inserted hCD2-LCR sequences in non-CD8-expressing hematopoietic cells indicates that the interaction of the LCR with gene-regulatory regions of the endogenous CD8 gene complex is restricted to cells descending from lymphoid lineage progenitors.

Taken together, our results showed that cells that have been affected by the inserted hCD2-LCR can convert to their destined fate upon deletion of the LCR. However, CD8 coreceptor-expression analyses in hematopoietic lineages suggest that the influence of inserted LCR sequences is largely dependent on chromatin accessibility of the CD8 gene complex in lymphoid cells. During early developmental stages (e.g., immature thymocytes) and during lineage commitment, when remodeling of the CD8 gene complex occurs, interference by the inserted LCR seems to be unrestricted; however, its influence seems to weaken throughout the life span of a cell.

Acknowledgments
We thank the members of the Divisions of Molecular Immunology and Immune Cell Biology at National Institute for Medical Research for helpful discussions. We are grateful to all of the members of the Flow Cytometry Facility and Biological Services at National Institute for Medical Research.

Disclosures
The authors have no financial conflicts of interest.

References
Figure S2

A  
peripheral CD8+ single positive T cells

mfi CD8α  
mfi CD8β

wt  cre-  cre+

0  10  20  30  40  50  60  70  80  90  100  110  120  130  140  150

0  10  20  30  40  50  60  70  80  90  100  110  120  130  140  150

wild type  CD8/ lxLCRlx+/cre-  CD8/ lxLCRlx+/cre+

B  
total thymus  total spleen (T cell enriched)  tail

deleted or wt allele  knock-in floxed allele

---

Figure S2

A  
peripheral CD8+ single positive T cells

mfi CD8α  
mfi CD8β

wt  cre-  cre+

0  10  20  30  40  50  60  70  80  90  100  110  120  130  140  150

0  10  20  30  40  50  60  70  80  90  100  110  120  130  140  150

wild type  CD8/ lxLCRlx+/cre-  CD8/ lxLCRlx+/cre+

B  
total thymus  total spleen (T cell enriched)  tail

deleted or wt allele  knock-in floxed allele

---
Figure S3

A

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<th>CD8/LCR3</th>
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B

Age related CD4+CD8+ expression

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<th>CD8/LCR3</th>
<th>wt</th>
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<tbody>
<tr>
<td>% CD4+CD8+ cells of total CD4+ cells</td>
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n(13) n(22) n(3) n(9) n(10) n(12) n(9) n(11) n(5) n(8) 3mo 7-12mo 17mo 2mo 7-12mo 14mo 2mo 9mo 14mo 3mo

Key:
- CD8/LCR
- CD8/LCR1,2
- CD8/LCR3
- wild type

**Statistical Significance:**
- *** p < 0.001
Figure S4

A

12 weeks after transfer

CD8/LCR

Input

Output

CD4

41%

59%

CD8α

CD8/LCR1,2

CD4

30%

63%

CD8α

B

% CD4+ CD8+ of total CD4+ cells

n=8

n=7

CD8/LCR

CD8/LCR1,2
FIGURE LEGENDS _ Supplementary Figures

Figure S1: CD8 co-receptor expression after developmental stage specific deletion of the inserted hCD2-LCR.

CD8 co-receptor expression in mature thymocytes (αβTCR^{high}) from CD8/lxLCRlx (cII-III) knock-in mice negative or positive for a Cre transgene analysed by plotting CD4 against CD8α. (A) vav-iCre. (B) hCD2-iCre. (C) dLck-iCre.

Figure S2: Cell lineage specific deletion of the inserted hCD2-LCR by dLck-iCre expression.

(A) Expression levels of the CD8 co-receptor in CD8^{+} single positive T cells measured by mean fluorescence intensity (mfi). Percentages are expressed in relation to wild type expression levels. Error bars show standard deviation (sd). Statistical analysis: unpaired Student’s T-test (**p \leq 0.001, ***p \leq 0.01). Number of mice analysed in each group (n).

(B) Tissue and stage specific deletion of the inserted hCD2-LCR by dLck-iCre expression. Southern blot analysis of total thymus cells, T cell enriched spleen cells and tail tissue from CD8/lxLCRlx knock-in mice negative or positive for the dLck-iCre transgene. The band for the wild type or deleted allele (13 kb) and the band for the knock-in floxed allele (4.4 kb) are indicated by arrows.

Figure S3: CD8 co-receptor expression in CD8/LCR knock-in mouse lines.

(A) Representative FACS blots of CD8α and CD8β expression in γδTCR^{+} lymphocytes from thymus or spleen.
(B) Age related influence on CD8 co-receptor expression. Comparison of CD8 co-receptor expression in peripheral T cells from young (≤ 3 months) and old (7-12 months or ≥ 14 months) CD8/LCR knock-in mice. Percentage of CD4⁺ CD8⁻ cells are shown in relation to total CD4⁺ T cells. Error bars show standard deviation. Number of mice analysed in each age group (n).

**Figure S4: Transfer of sorted lymphocytes cells from CD8/LCR knock-in mouse lines.**
Sorted peripheral CD4⁺(CD8⁺) lymphocytes from either CD8/LCR (cII-III) or CD8/LCR1,2 knock-in mice were transferred into lymphopenic hosts (Input). CD8 co-receptor expression profile of donor derived T cells 12 weeks after transfer (Output). Percentages indicated are in relation to total CD4⁺ cells.