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Coreceptor gene imprinting governs thymocyte lineage fate

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Immature thymocytes are bipotential cells that are signalled during positive selection to become either helper- or cytotoxic-lineage T cells. By tracking expression of lineage determining transcription factors during positive selection, we now report that the *Cd8* coreceptor gene locus co-opts any coreceptor protein encoded within it to induce thymocytes to express the cytotoxic-lineage factor Runx3 and to adopt the cytotoxic-lineage fate, findings we refer to as 'coreceptor gene imprinting'. Specifically, encoding CD4 proteins in the endogenous *Cd8* gene locus caused major histocompatibility complex class II-specific thymocytes to express Runx3 during positive selection and to differentiate into CD4$^+$ cytotoxic-lineage T cells. Our findings further indicate that coreceptor gene imprinting derives from the dynamic regulation of specific *cis Cd8* gene enhancer elements by positive selection signals in the thymus. Thus, for coreceptor-dependent thymocytes, lineage fate is determined by *Cd4* and *Cd8* coreceptor gene loci and not by the specificity of T-cell antigen receptor/coreceptor signalling. This study identifies coreceptor gene imprinting as a critical determinant of lineage fate determination in the thymus.

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Introduction

Cell-type diversity is achieved during development of multicellular organisms by extracellular signals that induce bipotential precursor cells to adopt alternative lineage fates. In the immune system, thymocytes at the CD4$^+$CD8$^+$ (double-positive, DP) stage of differentiation are bipotential cells that are signalled in the thymus by their T-cell antigen receptors (TCR) to undergo positive selection and to differentiate into functionally mature T cells possessing either helper or cytotoxic function. In most DP thymocytes, TCR signalling is dependent on CD4 or CD8 coreceptor proteins that bind to invariant portions of class II and class I major histocompatibility complex (MHC) determinants, respectively, with the result that CD4-dependent TCR engagement of class II MHC (MHCII) complexes signals DP thymocytes to differentiate into helper-lineage T cells, and CD8-dependent TCR engagement of class I MHC (MHCI) complexes signals DP thymocytes to differentiate into cytotoxic-lineage T cells (Starr *et al*, 2003). ThPOK has been identified as a transcription factor that specifies CD4 helper-lineage fate (He *et al*, 2005; Sun *et al*, 2005) and Runx3 as a transcription factor that specifies CD8 cytotoxic-lineage fate (Woolf *et al*, 2003; Sato *et al*, 2005; Chung *et al*, 2007; Egawa *et al*, 2007). MHCII-specific TCR/CD4 signalling normally results in thymocyte expression of ThPOK, whereas MHCI-specific TCR/CD8 signalling normally results in thymocytes expressing Runx3.

Despite the association of MHC specificity with lineage fate, there is compelling evidence that thymocyte expression of lineage-specific transcription factors is not due to the MHC specificity of the engaged TCR or the coreceptor proteins themselves. For example, induction of Runx3 expression in developing thymocytes is directly signalled by intrathymic cytokines but is not directly signalled by MHCI-specific TCR and CD8 coreceptors (Park *et al*, 2010). Importantly, the influence of endogenous *Cd4* and *Cd8* coreceptor gene loci on *in vivo* thymocyte lineage choice has never been examined and distinguished from that of the CD4/CD8 proteins they encode.

The present study has assessed the possibility that expression of lineage-specific transcription factors and thymocyte-lineage fate are determined by endogenous *Cd4/Cd8* gene loci regardless of the specific coreceptor protein each locus encodes. To do so, we varied the endogenous coreceptor gene locus in which CD4 proteins were encoded and observed that MHCII-selected thymocytes adopted entirely different lineage fates when their selection depended on CD4 coreceptor proteins encoded in *Cd8*, rather than *Cd4*, coreceptor gene loci. Specifically, CD4 coreceptor proteins encoded in *Cd8* gene loci promoted MHCII-specific thymocytes to express Runx3 (the cytotoxic-lineage transcription factor), not ThPOK (the helper-lineage transcription factor), resulting in their differentiation into CD4$^+$ cytotoxic-lineage T cells, not CD4$^+$ helper-lineage T cells. In fact, identical MHCII-specific thymocytes bearing identical transgenic TCR

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and identical CD4 proteins were found to express either ThPOK or Runx3, and to differentiate into either helper- or cytotoxic-lineage CD4$^+$ T cells, depending only on whether their CD4 proteins were encoded in *Cd4* or *Cd8* coreceptor gene loci. Thus, this study documents for the first time that endogenous *Cd4* and *Cd8* coreceptor gene loci encoding identical CD4 proteins induce MHCII-specific thymocytes to express different lineage-specific transcription factors and to adopt different lineage fates, findings we refer to as 'coreceptor gene imprinting'.

Results

Cd8a gene encoded CD4 proteins promote MHCII-specific positive selection

To change the coreceptor protein that the endogenous *Cd8* gene locus encoded, we used gene knock-in technology to replace CD8α-coding sequences with CD4 cDNA, generating a novel $Cd8a^{CD4}$ endogenous allele that encoded CD4 proteins instead of CD8α proteins (Supplementary Figure S1). Changing the coreceptor protein the *Cd8* gene locus encoded did not affect its expression pattern, as $Cd8a^{CD4}$-encoded CD4 proteins were expressed on CD8$^+$ thymocytes and peripheral CD8$^+$ T cells *in vivo* (Supplementary Figure S2A) and their expression was regulated by γ chain (γ_c)-dependent cytokines in parallel with CD8 proteins (Park *et al*, 2007) encoded by the wild-type $Cd8a^+$ endogenous allele (Supplementary Figure S2B). To generate mice whose CD4 proteins were only encoded in the *Cd8* gene locus, we bred the $Cd8a^{CD4}$ allele into $Cd4^{-/-}B2m^{-/-}$ mice to generate homozygous $Cd8a^{CD4/CD4}Cd4^{-/-}B2m^{-/-}$, referred to simply as '4in8 mice' (Figure 1A). 4in8 mice were genetically $Cd4^{-/-}$, so all CD4 proteins were encoded by their $Cd8a^{CD4}$ alleles; were genetically homozygous $Cd8a^{CD4/CD4}$ so CD8α proteins were not transcribed; and were genetically $B2m^{-/-}$ so T cells were only selected by MHCII selecting elements. MHCII-specific positive selection proceeded normally in 4in8 mice and was at least as efficient as MHCII-specific positive selection in conventional $B2m^{-/-}$ mice, since frequencies of TCRβ^{hi} thymocytes and numbers of peripheral TCRβ$^+$ lymph node (LN) T cells (50×10^6) were comparable (Figure 1A). MHCII expression was required for generation of 4in8 T cells because 4in8 mice lacking MHCII expression (4in8.MHCII$^{-/-}$) were devoid of positively selected TCRβ^{hi} thymocytes and so were additionally devoid of mature CD24^{lo}CD4$^+$ thymocytes and peripheral CD4$^+$TCRβ$^+$ T cells (Figure 1B). Thus, T cells generated in 4in8 and $B2m^{-/-}$ mice were identically MHCII specific, even though 4in8 mice genetically differed from conventional $B2m^{-/-}$ mice in the fact that their CD4 proteins were encoded in the *Cd8* gene locus instead of the *Cd4* gene locus.

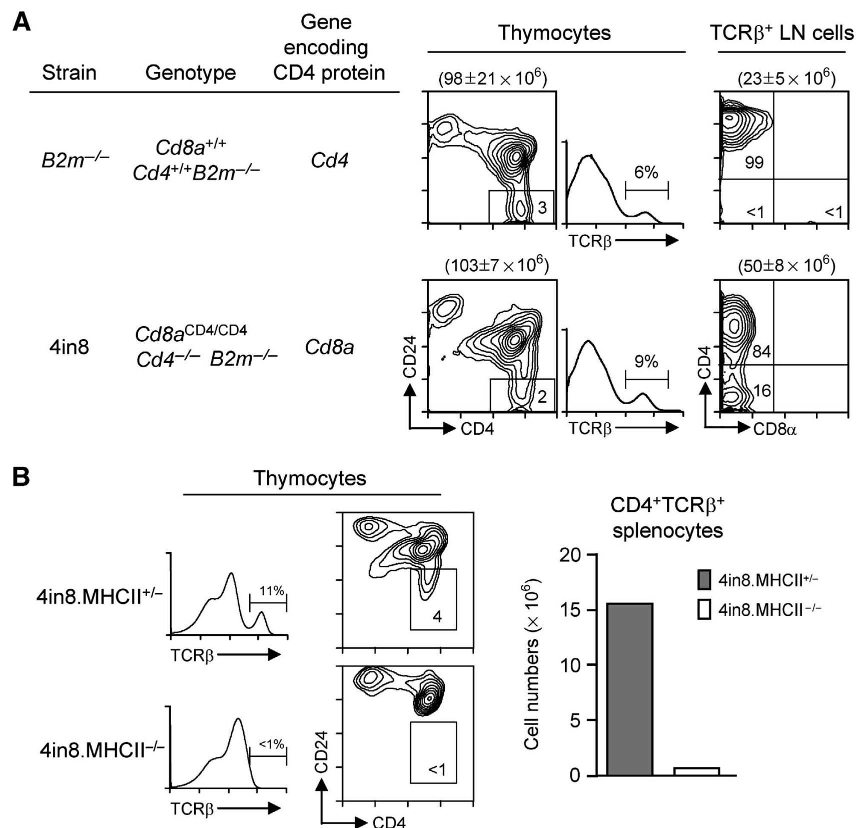

Figure 1 *Cd8*-encoded CD4 proteins promote MHCII-specific positive selection. (**A**) Analysis of MHCII-specific selection in $B2m^{-/-}$ and 4in8 ($Cd8a^{CD4/CD4}Cd4^{-/-}B2m^{-/-}$) mice. Shown are CD24 versus CD4 and TCRβ expression on total thymocytes (left panels) and CD4 versus CD8α expression on TCRβ$^+$ LN cells (right panels). Average (± s.e.m.; $n = 5$ mice per group) number of total thymocytes (left panel) and TCRβ$^+$ LN cell (right panel) are in parenthesis. Numbers in boxes or histograms indicate the frequency of cells within each gate. (**B**) TCRβ and CD4 versus CD24 expression on thymocytes from MHCII-deficient ($H2A\text{-}b1^{-/-}$) 4in8 mice (left panel) and absolute numbers of TCRβ$^+$CD4$^+$ splenocytes in MHCII-deficient 4in8 mice (right panel).

Coreceptor gene loci determines ThPOK versus Runx3 expression

To assess expression of lineage-specific factors during MHCII-specific positive selection, we identified MHCII-signalled CD69$^+$ thymocytes at progressive stages of development by differential surface expression of TCRβ and CD24 (Figure 2A, gates I*a*–IV*b*) as selected thymocytes gradually upregulate TCR as they downregulate CD24 (Bendelac *et al*, 1992). We obtained thymocytes from conventional *B2m*$^{-/-}$ and experimental 4in8 mice at each stage of differentiation by electronic sorting and assessed their expression of lineage-specific genes by quantitative real-time (qRT)–PCR (Figure 2B). The lineage-specific genes *Zbtb7b* and *Runx3* that encode ThPOK and Runx3 proteins, respectively, were not expressed in pre-selection (CD69$^-$TCR^{lo}CD24^{hi}) thymocytes from either *B2m*$^{-/-}$ or 4in8 mice, but were expressed in signalled CD69$^+$ thymocytes during positive selection. MHCII-signalled thymocytes from *B2m*$^{-/-}$ mice contained ThPOK mRNA, but not Runx3 mRNA. Remarkably, in contrast, MHCII-signalled thymocytes from 4in8 mice contained Runx3 mRNA, but little ThPOK mRNA (Figure 2B, left). Another gene whose expression differed between *B2m*$^{-/-}$ and 4in8 thymocytes was *Cd8b*, a cytotoxic-lineage gene, which was initially terminated during positive selection but was then selectively re-expressed in 4in8, not *B2m*$^{-/-}$, thymocytes (Figure 2B, left). Consequently, the lineage gene profile of MHCII-signalled 4in8 thymocytes (*Zbtb7b*^{lo} *Runx3*$^+$*Cd8b*$^+$) markedly differed from that of conventional MHCII-signalled *B2m*$^{-/-}$ thymocytes (*Zbtb7b*$^+$*Runx3*$^-$ *Cd8b*$^-$), which differentiate into helper-lineage T cells. However, the lineage gene profile of MHCII-signalled 4in8 thymocytes was nearly identical to that of conventional MHCI-signalled thymocytes (*Zbtb7b*$^-$*Runx3*$^+$*Cd8b*$^+$), which depend on *Cd8*-encoded CD8 proteins and differentiate into cytotoxic-lineage T cells (Figure 2B, right).

Interestingly, thymocyte mRNA levels of *Gata3* and *Tox*, which encode transcription factors primarily associated with CD4/helper-lineage choice (Hernandez-Hoyos *et al*, 2003; Aliahmad and Kaye, 2008), did not distinguish developing cytotoxic-lineage thymocytes from developing helper-lineage thymocytes, as *Gata3* and *Tox* expression diverged only in thymocytes at the most mature developmental stage, that is, CD69$^-$TCR^{hi}CD24^{lo}, after lineage fate had been decided (Figure 2B, lower panels).

Despite the markedly different lineage gene profiles for *Zbtb7b*, *Runx3*, and *Cd8b* displayed by 4in8 and *B2m*$^{-/-}$ thymocytes (*Zbtb7b*^{lo}*Runx3*$^+$*Cd8b*$^+$ versus *Zbtb7b*$^+$*Runx3*$^-$ *Cd8b*$^-$), CD4 proteins were expressed in comparable amounts in 4in8 and *B2m*$^{-/-}$ mice on the surface of pre- and post-selection thymocytes (Supplementary Figure S3), were equally associated with Lck kinase (Supplementary Figure S4A), and were equally competent to promote phosphorylation of TCR-associated substrates and TCR signalling

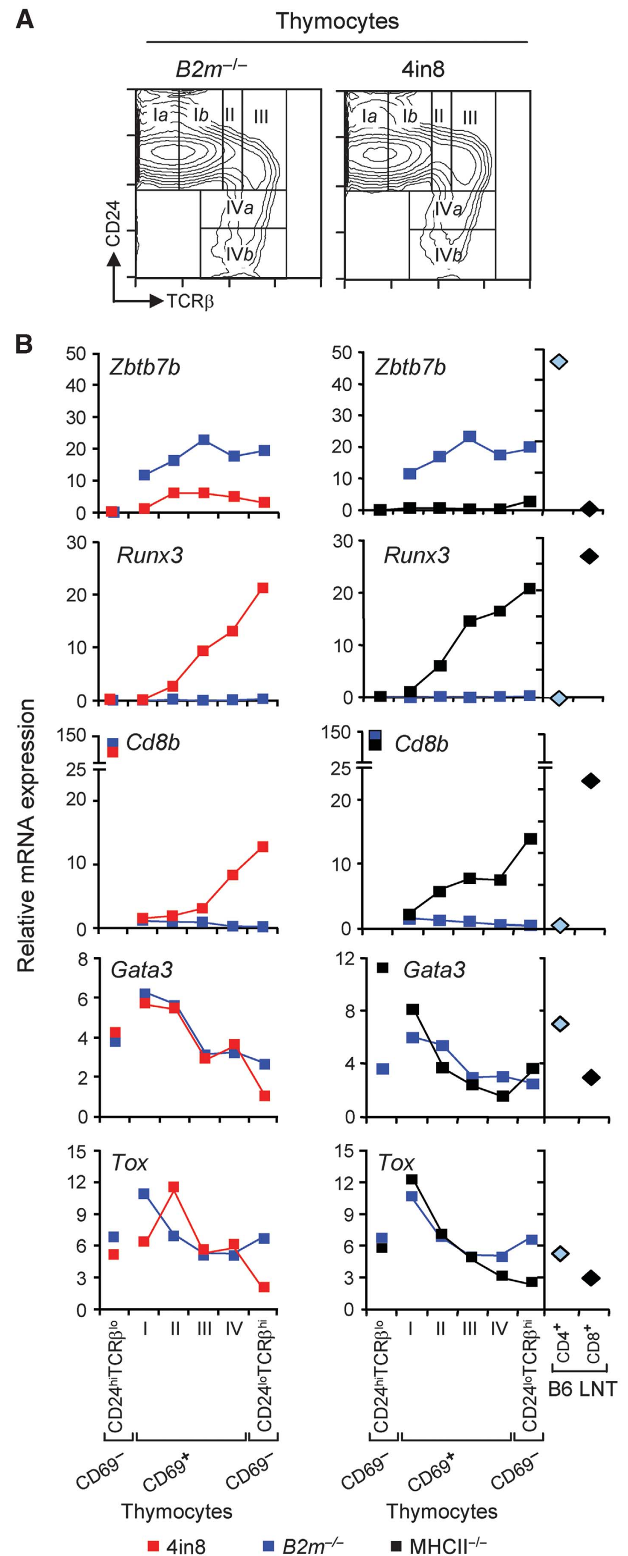

Figure 2 *Cd4* and *Cd8* coreceptor gene loci determine lineage-specific gene expression in positively selected thymocytes. (**A**) Gating strategy for identifying progressive stages of thymocyte development. TCRβ versus CD24 expression identifies progressive stages (I*a* thru IV*b*) of positive selection in total thymocytes. (**B**) Quantitative RT–PCR analyses of genes encoding ThPOK (*Zbtb7b*), *Runx3*, *Cd8b*, *Gata3*, and *Tox* (normalized to β-actin mRNA) in developing thymocytes. Thymocytes were sorted based on surface expression of CD69, CD24, and TCRβ. Pre-selection thymocytes were CD69$^-$CD24^{hi}TCR^{lo}; recently signalled thymocytes were CD69$^+$CD24^{hi}TCR^{lo} (gate I); thymocytes undergoing positive selection were CD69$^+$CD24^{hi}TCR$^{int/hi}$ (gates II and III); thymocytes maturing into single positive (SP) T cells were CD69$^+$CD24^{lo}TCR^{hi} (gate IV); and the most mature SP thymocytes were CD69$^-$ CD24$^{lo/-}$TCR^{hi}. As positive controls, mRNA levels of the indicated genes in B6 CD4$^+$ and CD8$^+$ LN T cells are shown. Data are representative of two independent experiments.

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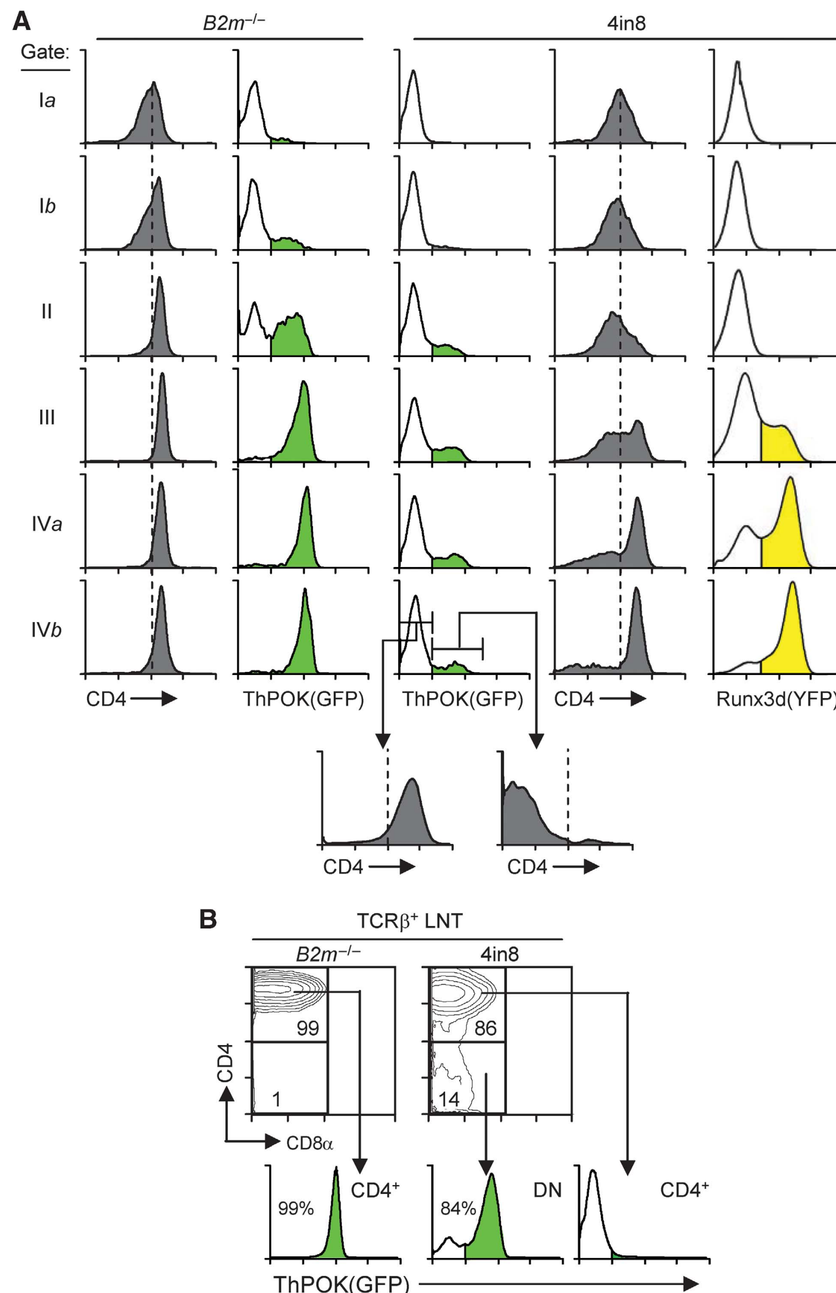

Figure 3 Tracking ThPOK and Runx3 expression in MHCII-selected thymocytes. (**A**) Expression of CD4, ThPOK(GFP) and Runx3d(YFP) reporters in wild-type (*B2m*$^{-/-}$) and 4in8 signalled thymocytes at progressive stages of development: gate I*a* (CD69$^+$CD24^{hi}TCR^{lo}); gate I*b* (CD69$^+$CD24^{hi}TCR$^{lo/int}$); gate II (CD69$^+$CD24^{hi}TCR^{int}); gate III (CD69$^+$CD24^{hi}TCR^{hi}); gate IV*a* (CD69$^+$CD24^{lo}TCR^{hi}); gate IVb (CD69$^+$CD24$^-$TCR^{hi}). Dashed vertical lines indicate the peak of CD4 expression on pre-selection DP thymocytes. GFP$^+$ cells are shaded green and YFP$^+$ cells are shaded yellow. (**B**) ThPOK(GFP) expression (GFP$^+$ cells are shaded green) on TCRβ$^+$ LN T cells from *B2m*$^{-/-}$ and 4in8 mice. The frequency of cells in each gate is shown. Data are representative of two independent experiments.

(Supplementary Figure S4B and C) and calcium mobilization (Supplementary Figure S5). Thus, it is the *Cd4* and *Cd8* coreceptor gene loci, not the coreceptor proteins they encode nor the strength of coreceptor protein signalling, that influence which lineage-specific genes positively selected thymocytes express.

Cd8a-encoded CD4 proteins promote Runx3 induction

To determine the mechanism by which *Cd4* and *Cd8* coreceptor gene loci influenced ThPOK and Runx3 lineage factor expression in signalled thymocytes, we compared expression of *Cd4*-encoded and *Cd8*-encoded CD4 proteins during positive selection of thymocytes expressing either a ThPOK(GFP) reporter (Wang *et al*, 2008) or a Runx3d(YFP) reporter (Egawa and Littman, 2008) (Figure 3A). Among conventional *B2m*$^{-/-}$ thymocytes whose CD4 proteins were encoded in *Cd4* gene loci, positive selection signals progressively increased CD4 and ThPOK(GFP) expression until all MHCII-signalled thymocytes differentiated into CD4$^+$ThPOK (GFP)$^+$ mature T cells (Figure 3A, columns 1 and 2). In contrast, among 4in8 thymocytes whose CD4 proteins were encoded in *Cd8* gene loci, positive selection signals initially

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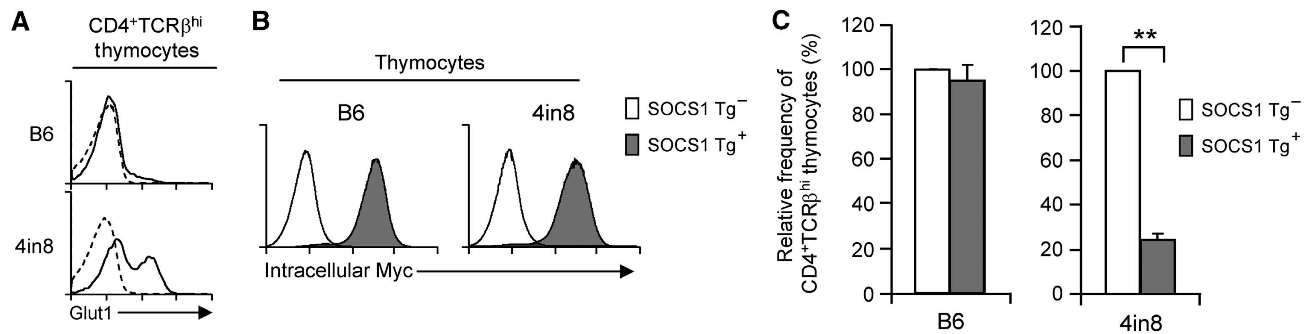

Figure 4 Cytokine signals regulate differentiation of thymocytes selected by *Cd8*-encoded CD4. (**A**) Glut1 expression (solid line) on $CD4^+TCR\beta^{hi}$ thymocytes from B6 and 4in8 mice. Dashed line represents control antibody staining. Data are representative of three experiments. (**B**) Expression of myc-tagged SOCS1 transgene in wild-type (B6) and 4in8 mice assessed by intracellular staining for myc. Shaded histograms represent myc expression in transgenic mice and unshaded histograms are control staining with the same anti-myc antibody in non-transgenic mice. (**C**) Frequency (average ± s.e.m.; $n = 3$ mice per group) of $CD4^+TCR\beta^{hi}$ thymocytes in SOCS1 transgenic mice (SOCS1 Tg) relative to non-transgenic mice (100%). $^{**}P<0.005$ (Student's *t*-test).

downregulated CD4 protein expression (Figure 3A, column 4) and led to the generation of two distinct thymocyte subpopulations: (1) a major subpopulation (~85%) of signalled thymocytes that re-expressed their CD4 proteins, along with Runx3(YFP) and differentiated into $CD4^+ThPOK(GFP)^-$ mature T cells that were $Runx3(YFP)^+$ (Figure 3A, columns 3–5) and (2) a minor subpopulation (~15%) of signalled thymocytes (which likely account for the low-level ThPOK mRNA in sorted 4in8 thymocytes; Figure 2B) that did not re-express CD4 but did express ThPOK(GFP) and differentiated into $CD4^-ThPOK(GFP)^+$ mature T cells that were $Runx3(YFP)^-$ (Figure 3A, columns 3–5). Both $CD4^+ThPOK(GFP)^-$ and $CD4^-ThPOK(GFP)^+$
T-cell subpopulations in the thymus emigrated into the periphery where they were found in 4in8 mice in the same relative proportion as in the thymus (Figure 3B).

To first understand the generation of the major subpopulation of $CD4^+ThPOK^-$ T cells in 4in8 mice, we considered that the transient downregulation of *Cd8*-encoded CD4 proteins during positive selection (Figure 3A, column 4) would disrupt, albeit transiently, MHCII-specific TCR signalling in CD4-dependent thymocytes (Sarafova *et al*, 2005, 2009). We further considered that disruption of TCR signalling during positive selection permits thymocytes to be signalled by intrathymic cytokines, which induces Runx3 expression (Park *et al*, 2010) and transcriptionally silences the *Zbtb7b* gene to prevent ThPOK expression (Setoguchi *et al*, 2008). Consequently, we examined if $CD4^+Runx3^+$ T-cell generation in 4in8 mice was the result of intrathymic cytokine signalling. Consistent with their being cytokine signalled, $CD4^+TCR\beta^{hi}$ thymocytes from 4in8 mice (but not conventional mice) expressed Glut1 (Figure 4A), a downstream target of IL-7 signalling (Yu *et al*, 2003; Wofford *et al*, 2008). To determine if $CD4^+$ T-cell generation in 4in8 mice actually required cytokine signalling, we introduced a transgene encoding myc-tagged suppressor of cytokine signalling 1 (SOCS1) protein under the control of the proximal Lck promoter (Hanada *et al*, 2001) to inhibit cytokine signal transduction in developing thymocytes (Figure 4B). Importantly, the SOCS1 transgene did significantly inhibit thymic generation of $CD4^+$ T cells in 4in8 mice, but not in wild-type mice (Figure 4C). These results demonstrate that cytokine signalling is required for the positive selection of thymocytes dependent on CD4 proteins that are encoded in the *Cd8* gene locus, but is not required if CD4 proteins are encoded in the *Cd4* gene locus. Consequently, because cytokine signalling induces Runx3, which silences ThPOK expression (Setoguchi *et al*, 2008), the lineage-specific factor that CD4-dependent thymocytes express during MHCII-specific positive selection depends on which coreceptor gene locus encodes the CD4 proteins. Thus, *Cd4*-encoded CD4 proteins promote ThPOK expression, whereas *Cd8*-encoded CD4 proteins promote Runx3 expression.

Impact of positive selection signals on cis regulatory elements in the Cd8 gene

Although MHCII-specific positive selection signals upregulated expression of *Cd4*-encoded CD4 proteins, the same MHCII-specific positive selection signals downregulated expression of *Cd8*-encoded CD4 proteins (Figures 3A and 5A), with the result that positively selected thymocytes were signalled by intrathymic cytokines to express Runx3 and to differentiate into cytotoxic-lineage $CD4^+$ T cells. Consequently, we assessed which *cis* regulatory elements in the *Cd8* gene locus were responsible for transient downregulation of *Cd8*-encoded CD4 proteins during positive selection. As schematized in Figure 5B, three tissue-specific enhancer elements have been identified in the *Cd8* gene ($E8_{III}$, $E8_{II}$, and $E8_{I}$) that regulate expression of *Cd8*-encoded coreceptor proteins during thymocyte development (Ellmeier *et al*, 1999; Kioussis and Ellmeier, 2002), but it is unclear how their activities are orchestrated during positive selection to transiently terminate expression of *Cd8*-encoded CD4 coreceptor proteins. It is known that the $E8_{III}$ and $E8_{II}$ enhancers are both active in pre-selection DP thymocytes (Ellmeier *et al*, 1998; Feik *et al*, 2005), and that the $E8_{II}$ and $E8_{I}$ enhancers are both active in post-selection T cells (Ellmeier *et al*, 1997, 2002; Hostert *et al*, 1997). However, it is difficult to reconcile the fact that the $E8_{II}$ enhancer is active in both pre- and post-selection thymocytes with the transient termination of *Cd8* gene expression during positive selection. Importantly, the potential effect of positive selection signalling on $E8_{II}$ enhancer activity has never been examined. Consequently, we constructed three transgenes (referred to as $E8_{III}$-CD4, $E8_{II}$-CD4, and $E8_{I}$-CD4) that encode CD4 proteins under the control of individual *Cd8* enhancer/pro-

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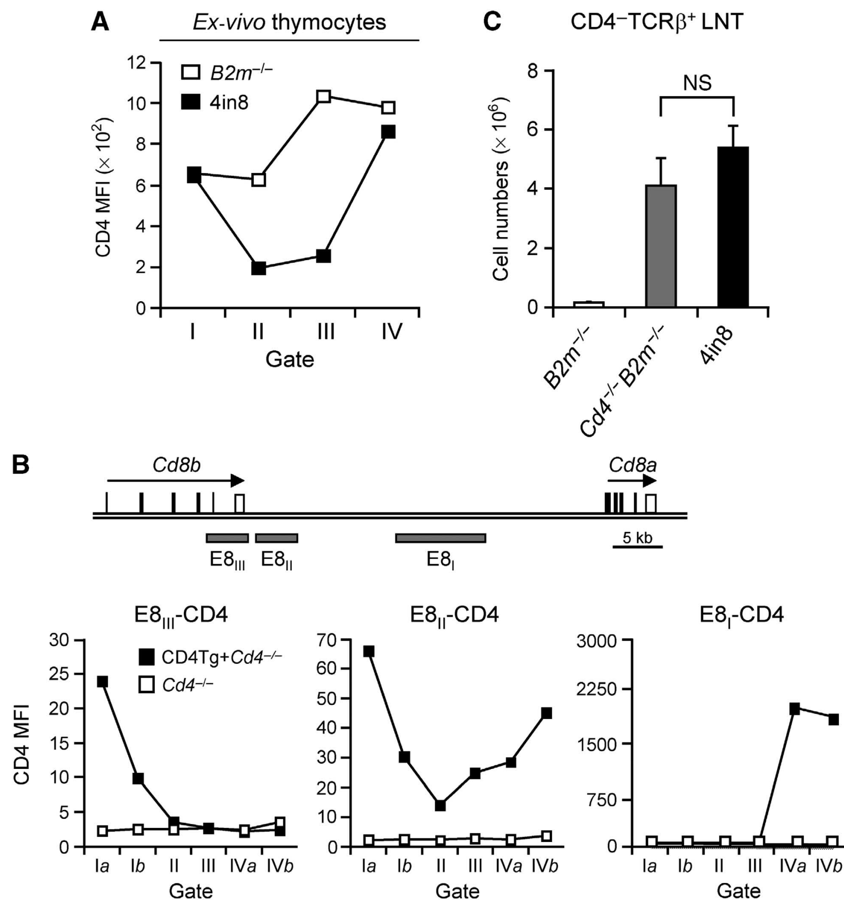

Figure 5 Dynamic regulation of the activity of *Cd8* gene *cis* enhancers by positive selection signals. (**A**) Mean fluorescence intensity (MFI) of surface CD4 expression on progressive stages of thymocyte development (I thru IV) defined by CD24 and TCRβ expression as in Figure 2A. (**B**) *In-vivo* activity of *cis Cd8* gene enhancers. (Top) Schematic of the endogenous *Cd8* gene locus showing position of *cis* regulatory enhancers. (Bottom) $E8_{III}$, $E8_{II}$, and $E8_{I}$ regulated CD4 expression in developing thymocytes. Graphs show the MFI of CD4 determined by flow cytometry on thymocyte populations gated as shown in Figure 2A. Note that all three CD4 transgenes were expressed in CD4-deficient (*Cd4*$^{-/-}$) mice so that all CD4 expression was regulated by transgenic enhancers. Data are representative of two or three independent experiments. (**C**) Coreceptor-independent MHCII-selected T cells in the indicated mice. Data are average (±s.e.m.) of $CD4^{-}TCR\beta^{+}$ LN T cells in the indicated mice. NS, not significant (Student's *t*-test); $n=5$ mice per group.

moter regulatory elements (Supplementary Figure S6) and expressed the transgenes in *Cd4*$^{-/-}$ mice so that we could assess individual *Cd8* enhancer activity throughout positive selection (Figure 5B). As revealed by CD4 protein expression, we confirmed that $E8_{III}$ and $E8_{II}$ enhancers were both active in pre-selection thymocytes (Figure 5B, gates I*a*, I*b*), and that $E8_{II}$ and $E8_{I}$ enhancers were both active in post-selection thymocytes (Figure 5B, gates IV*a*, IV*b*). Importantly, however, we observed that the $E8_{II}$ enhancer was terminated by positive selection signalling (Figure 5B, gates II and III), but only transiently, as $E8_{II}$ enhancer activity was re-expressed. Thus, transient termination of *Cd8*-encoded CD4 coreceptor proteins during positive selection of 4in8 thymocytes specifically reflects the carefully choreographed and differential effects of positive selection signals on $E8_{III}$, $E8_{II}$, and $E8_{I}$ enhancer elements in the *Cd8* gene locus.

Coreceptor-independent MHCII-selected T cells

To then understand the generation of the minor $CD4^{-}$ $ThPOK^{+}$ T-cell subpopulation in 4in8 mice, we compared 4in8 mice (which were genetically *Cd8a*$^{CD4/CD4}$*Cd4*$^{-/-}$ *B2m*$^{-/-}$) with mice that were genetically *Cd8a*$^{+/+}$ *Cd4*$^{-/-}$*B2m*$^{-/-}$. Both of these mouse strains were *Cd4*$^{-/-}$ and contained only MHCII-selected T cells, so the key difference between them was whether their T cells expressed *Cd8*-encoded CD4 proteins or no CD4 proteins at all. We found comparable numbers of mature $CD4^{-}$ T cells in the periphery of both mouse strains (Figure 5C), regardless of whether CD4 proteins were present or absent, revealing that the generation of such $CD4^{-}$ T cells was CD4 independent. Thus, $CD4^{-}ThPOK^{+}$ T cells arose in 4in8 mice, despite *Cd8*-encoded CD4 proteins, because the generation of these T cells was CD4 independent. Taken together, these results reveal that thymocytes whose positive selection depend on *Cd8*-encoded coreceptor proteins express Runx3, regardless of the MHC specificity of their TCR and regardless of which coreceptor protein they express.

Repertoire and specificity of T cells dependent on Cd8a-encoded CD4 proteins

To understand whether *Cd8a*-encoded CD4 promoted normal MHCII-specific selection or merely permitted the differentiation of a subset of T cells, we next analysed the TCR specificity of mature T cells in the periphery. In 4in8 mice, TCR-Vα and TCR-Vβ gene usage by $CD4^{+}$ T cells resembled that of conventional $CD4^{+}$ T cells, but differed significantly

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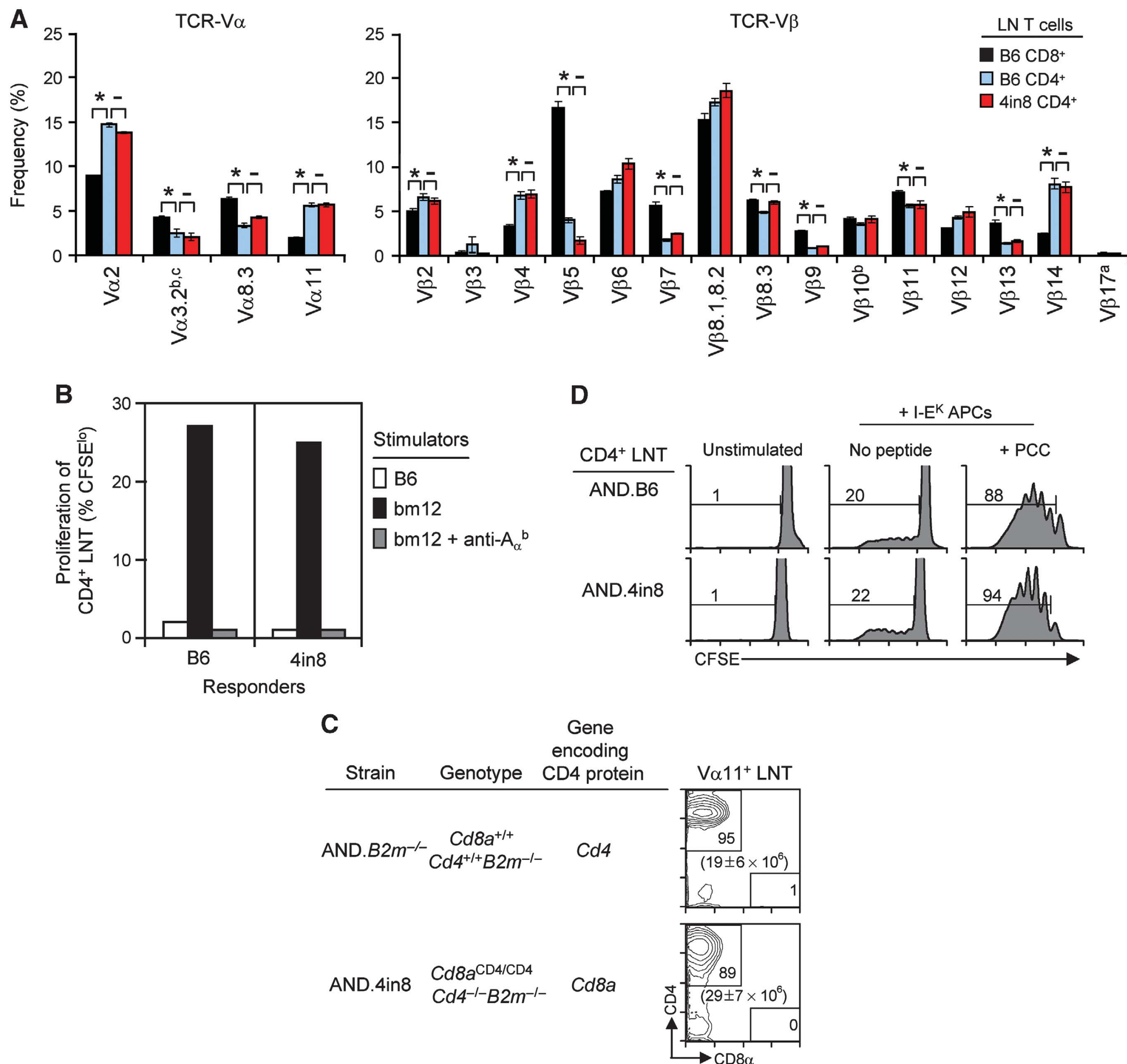

Figure 6 MHCII specificity of 4in8 T cells. (**A**) TCR-Vα and TCR-Vβ usage by LN T cells from B6 and 4in8 ($n=3$ mice per group; error bar, s.e.m.; –, not significant; *$P<0.01$, Student's *t*-test). (**B**) Frequency of proliferated (>1 cell division) LN cells after stimulation of CFSE-labelled CD4$^+$ LN T cells from B6 and 4in8 mice with syngeneic B6 or MHCII-disparate bm12 splenocytes in the presence or absence of anti-Aα^b (Y3P) antibody for 4 days. (**C**) Vα11$^+$ LN T cells from *B2m*$^{-/-}$ and 4in8 mice expressing the AND (Vα11$^+$Vβ3$^+$) TCR transgene. Absolute numbers (average ± s.e.m.; $n=5$ mice per group) of Vα11$^+$CD4$^+$ LN cells are indicated in parenthesis and the frequency of cells within each gate is indicated. (**D**) Reactivity of CD4$^+$ T cells from wild-type (AND.B6) and 4in8 (AND.4in8) mice expressing the AND TCR transgene to PCC presented by B10.A (I-E^k) splenocytes. Histograms show CFSE expression in stimulated CD4 T cells and numbers indicate the frequency of cells with >1 division. Data are representative of three independent experiments.

from that of conventional CD8$^+$ T cells, indicating that the TCR specificities of MHCII-specific T cells were not affected by the coreceptor gene locus in which CD4 proteins were encoded (Figure 6A). This finding was also supported by the fact that both 4in8 and B6 CD4$^+$ T cells reacted against bm12 stimulator cells that express mutant Aβ^{bm12} MHCII molecules (McKenzie *et al*, 1979), and their reactivities were equally blocked by MHCII-specific anti-Aα^b monoclonal antibody (Figure 6B; Supplementary Figure S7A). Notably, their similar TCR specificities did not result from homeostatic expansion of CD4$^+$ T cells in either 4in8 or B6 mice, as both CD4$^+$ T-cell populations consisted primarily of phenotypically naive CD69$^-$CD44^{lo}CD62L^{hi} cells (Supplementary Figure S7B).

To confirm with a fixed monoclonal TCR that the *Cd8* coreceptor gene locus did not affect the selection of MHCII-specific TCR, we introduced the MHCII-restricted AND (Vα11$^+$Vβ3$^+$) TCR transgene (Kaye *et al*, 1989) into both 4in8 and conventional *B2m*$^{-/-}$ mice, generating AND.4in8 and AND.*B2m*$^{-/-}$ mice (Figure 6C; Supplementary Figure S8). Importantly, CD4$^+$ T cells from both AND.4in8 and AND.*B2m*$^{-/-}$ mice proliferated against the agonist ligand for the AND TCR, which is pigeon cytochrome c (PCC) peptide presented by I-E^k splenocytes (Figure 6D). The low-level proliferation detected without PCC peptide reflected AND TCR reactivity against allogeneic I-E^k determinants (Figure 6D, middle). Thus, CD4 proteins promoted the selection and differentiation of thymocytes with similar, if not identical, MHCII-specific TCRs, regardless of the coreceptor gene locus in which the CD4 proteins were encoded.

Lineage fate and function of 4in8 T cells

We then assessed the effect of coreceptor gene loci on the lineage fate adopted by mature CD4$^+$ T cells. The lineage-specific gene profile expressed by CD4$^+$ LN T cells

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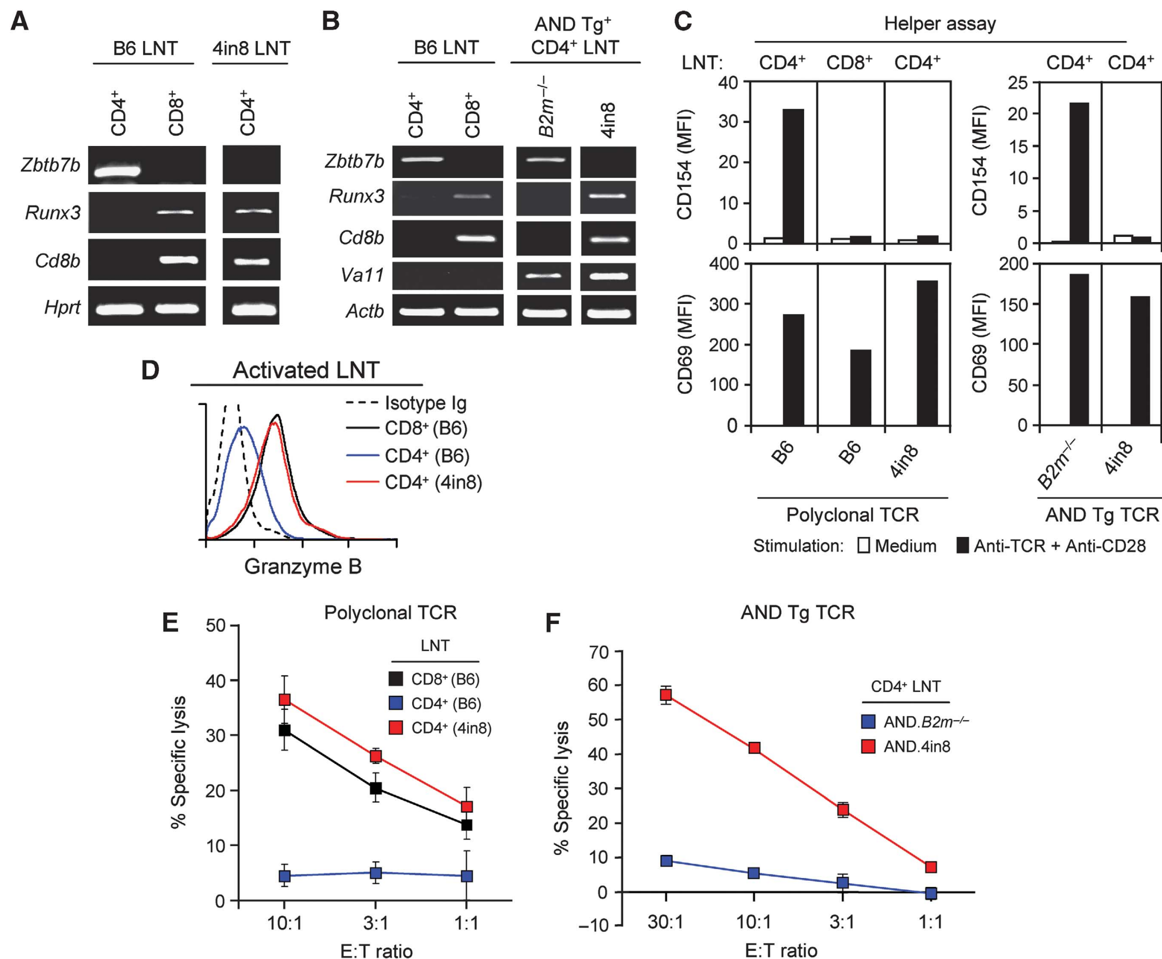

Figure 7 MHCII-selected 4in8 CD4$^+$ T cells are cytotoxic-lineage T cells. (**A**, **B**) RT–PCR analyses of mRNA expression in sorted polyclonal (**A**) or AND TCR transgenic (**B**) B6 and 4in8 LN T-cell subsets. (**C**) Mean fluorescence intensity (MFI) of CD154 and CD69 expression on polyclonal (left) or AND TCR transgenic (right) B6 and 4in8 LN T cells after 12 h stimulation. (**D**) Intracellular granzyme B expression in effector B6 CD4$^+$, B6 CD8$^+$, and 4in8 CD4$^+$ LN T cells. (**E**, **F**) Re-directed cytotoxic activity of polyclonal (**E**) or AND TCR transgenic (**F**) effector LN T cells against ^{51}Cr-labelled Fas-deficient L1210 target cells at varying effector–target (E:T) ratios. Each data point represents the mean (±s.d.) of triplicate cultures. Data are representative of at least three independent experiments.

from either 4in8 or AND.4in8 mice (*Zbtb7b*$^-$*Runx3*$^+$*CD8b*$^+$) was opposite to that of conventional CD4$^+$ helper-lineage T cells (*Zbtb7b*$^+$*Runx3*$^-$*CD8b*$^-$), but resembled that of conventional CD8$^+$ cytotoxic-lineage T cells (*Zbtb7b*$^-$*Runx3*$^+$ *CD8b*$^+$) (Figure 7A and B).

To determine the function of 4in8 CD4$^+$ T cells, we first assessed their helper function by upregulation of CD154 (CD40 ligand) expression (Noelle *et al*, 1992). Unlike conventional CD4$^+$ T cells that upregulated CD154 in response to TCR/CD28 stimulation, CD4$^+$ T cells from 4in8 and AND.4in8 mice did not upregulate CD154 in response to TCR/CD28 stimulation, even though they were signalled to upregulate CD69, revealing that they lacked helper function (Figure 7C). In addition, 4in8 CD4$^+$ T cells were unable to provide help for *in vivo* T-dependent antibody responses (SA, TM, and AS, unpublished observation). Although 4in8 CD4$^+$ T cells lacked helper function, they did possess cytotoxic function, as anti-TCR/CD28 stimulation induced 4in8 CD4$^+$ T cells to express the cytotoxic-lineage marker granzyme B (Figure 7D) and to become functionally cytolytic cells. Indeed, following TCR stimulation, both 4in8 and 4in8.AND CD4$^+$ T cells killed Fas-deficient L1210 target cells in a TCR-dependent re-directed cytotoxicity assay (Figure 7E and F). MHCII-specific 4in8 CD4$^+$ T cells also functionally resembled conventional MHCI-specific CD8$^+$ T cells in their preferential secretion of IFNγ, not IL-4, in response to TCR/CD28 stimulation under Th2 polarization conditions (Supplementary Figure S9). Thus, despite their expression of similar MHCII-restricted TCR specificities as conventional CD4$^+$ T cells, 4in8 CD4$^+$ T cells were functionally cytotoxic-lineage T cells. These results reveal that encoding CD4 proteins in the *Cd8* coreceptor gene locus had no effect on the MHCII specificity of CD4-dependent T cells but completely altered the lineage fate they adopted.

Discussion

This study reports the discovery of 'coreceptor gene imprinting' by revealing that the *Cd8* coreceptor gene locus co-opts any coreceptor protein encoded within it to induce positively selected thymocytes to express Runx3 and to adopt the cytotoxic-lineage fate. Indeed, thymocytes whose positive selection is dependent on *Cd8*-encoded coreceptor proteins were signalled by intrathymic cytokines to express Runx3 and

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to differentiate into cytotoxic-lineage T cells, regardless of the MHC specificity of their TCR and regardless of the coreceptor protein they express. The basis for coreceptor gene imprinting by the *Cd8* gene locus derives from the transient termination of *Cd8* gene transcription and initial downregulation of *Cd8*-encoded coreceptor proteins during positive selection.

To reveal coreceptor gene imprinting, it was necessary to construct novel 4in8 mice whose endogenous *Cd8a* genes were re-engineered to encode CD4 coreceptor proteins. Notably, our knock-in strategy did not affect any of the known *cis* regulatory elements in the *Cd8* gene locus, which are all located upstream of exon I and remained intact in the engineered $Cd8a^{CD4}$ locus (Ellmeier *et al*, 1999; Kioussis and Ellmeier, 2002), although it remained formally possible that we disrupted important but unknown intronic regulatory elements between exons I–V of the *Cd8* gene locus. However, contrary to this possibility, CD4 proteins encoded by re-engineered $Cd8a^{CD4}$ genes and CD8 proteins encoded by endogenous $Cd8a^{+}$ genes were identically expressed on cells in the thymus and periphery and responded identically to stimulatory cytokines.

Examination of 4in8 mice revealed two phenotypically and functionally distinct populations of MHCII-selected T cells: a major population (85%) whose positive selection in the thymus was CD4 dependent and which differentiated into mature $CD4^{+}$ T cells with matching TCR/CD4 specificities that were unique in that they possessed cytotoxic function; and a minor population (15%) whose positive selection in the thymus was coreceptor independent and which differentiated into mature DN T cells that expressed ThPOK. Both 4in8 T-cell populations significantly contribute to our understanding of lineage fate determination in the thymus. The major 4in8 T-cell population (whose selection in the thymus is CD4 dependent and which differentiate into $CD4^{+}$ cytotoxic-lineage T cells) demonstrates that strong signalling from matching MHCII-specific TCR and CD4 coreceptor proteins neither prevents adoption of the cytotoxic-lineage fate nor promotes adoption of the helper-lineage fate. Instead, the lineage fate adopted by CD4-dependent thymocytes is determined by the coreceptor gene locus in which CD4 proteins are encoded. And the minor 4in8 T-cell population (whose selection in the thymus is CD4 independent and which differentiate into phenotypically distinct DN T cells) reveals that ∼15% of MHCII-specific thymic selection is CD4 coreceptor independent. Indeed, it is only in 4in8 mice that CD4-dependent and CD4-independent thymocytes differentiate into phenotypically different T-cell subsets, because in wild-type mice MHCII-specific thymocytes differentiate into identical $CD4^{+}$ T cells, regardless of whether their selection was coreceptor dependent or independent.

Surface expression of *Cd4*-encoded CD4 proteins increased on TCR-signalled thymocytes during MHCII-specific positive selection, which would promote CD4-dependent TCR signalling to persist, whereas surface expression of *Cd8*-encoded CD4 proteins decreased which would cause CD4-dependent TCR signalling to be disrupted. Because these different expression kinetics led the same MHCII-specific and CD4-dependent thymocytes to adopt different lineage fates, the present study are consistent with the concept that the persistence or disruption of TCR-mediated positive selection signalling is the critical factor for lineage fate determination in the thymus (Singer, 2002; Singer *et al*, 2008). Indeed, CD4-dependent thymocytes that experienced persistent positive selection signalling expressed ThPOK, not Runx3, and their expression of ThPOK steadily increased during differentiation into mature helper T cells; whereas CD4-dependent thymocytes that experienced disrupted positive selection signalling expressed Runx3, not ThPOK, and their expression of Runx3 steadily increased during differentiation into mature cytotoxic T cells. Thus, persistent *in vivo* positive selection signalling induces ThPOK expression, whereas disrupted *in vivo* positive selection signalling results in Runx3 expression. Interestingly, expression of ThPOK or Runx3 was mutually exclusive in positively selected thymocytes, which is consistent with each factor antagonizing each other's transcription (Egawa and Littman, 2008; He *et al*, 2008; Setoguchi *et al*, 2008).

That ThPOK expression is induced by persistent *in vivo* positive selection TCR signalling is further supported by our analysis of the minor population of coreceptor-independent thymocytes in 4in8 mice. Unlike CD4-dependent TCR signalling, which is disrupted during MHCII-specific positive selection in 4in8 mice by declining expression of *Cd8*-encoded CD4 coreceptor proteins, CD4-independent MHCII-specific TCR signalling persists throughout positive selection. Because CD4 protein downregulation during positive selection would not disrupt MHCII-specific TCR signalling in CD4-independent thymocytes, TCR signalling would be persistent and prevent cytokine signalling, would induce ThPOK expression, and would terminate expression of *Cd8*-encoded genes (Egawa and Littman, 2008; Muroi *et al*, 2008; Singer *et al*, 2008), explaining the differentiation of CD4-independent thymocytes in 4in8 mice into $CD4^{-}ThPOK^{+}$ T cells. Accordingly, we found that CD4-independent thymocytes in 4in8 mice expressed ThPOK during MHCII-specific positive selection and differentiated into helper-lineage T cells. Thus, coreceptor-independent thymocytes in 4in8 mice provide independent confirmation that persistent *in vivo* positive selection signalling is responsible for inducing ThPOK expression (He *et al*, 2008; Singer *et al*, 2008), and emphasize that lineage fate determination for coreceptor-independent thymocytes differs from that for coreceptor-dependent thymocytes in being unaffected by the kinetics of coreceptor protein expression.

In addition to ThPOK, Gata3 and Tox transcription factors have been shown to be important for differentiation of MHCII-selected thymocytes into $CD4^{+}$ T-helper cells (Hernandez-Hoyos *et al*, 2003; Aliahmad and Kaye, 2008). However, both GATA3 and Tox were comparably expressed throughout MHCII-specific positive selection, diverging only in the most mature ($CD69^{-}CD24^{lo}TCR^{hi}$) thymocyte subset in 4in8 and wild-type mice, regardless of whether thymocytes were differentiating into cytotoxic- or helper-lineage T cells. Indeed, at no point in positive selection did expression of either *Gata3* or *Tox* distinguish MHCII-signalled thymocytes that were differentiating into $CD4^{+}$ T-helper cells from MHCII-signalled thymocytes that were differentiating into $CD4^{+}$ T-cytotoxic cells. Consequently, we conclude that expression of GATA3 and/or Tox neither identifies nor determines thymocyte-lineage fate, even though they are necessary cofactors for CD4/helper-lineage fate specification (Aliahmad and Kaye, 2008; Wang *et al*, 2008).

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Disruption of TCR signalling during positive selection is thought to be critical for thymocyte adoption of the cytotoxic-lineage fate because it is only by disrupting TCR signalling that positively selected thymocytes can be signalled by intrathymic cytokines to express Runx3 (Singer *et al*, 2008; Park *et al*, 2010). The results of our present study are consistent with this concept as SOCS1-induced blockade of cytokine signalling specifically impaired differentiation of cytotoxic-lineage but not helper-lineage $CD4^+$ T cells. Additionally, the present results indicate that transient coreceptor downregulation, which would cause transient disruption of TCR signalling, is sufficient to induce Runx3 expression and to irreversibly repress ThPOK, leading to differentiation into cytotoxic-lineage T cells. In thymocytes from 4in8 mice, expression of *Cd8*-encoded CD4 molecules declined during positive selection until CD4-dependent signalling was disrupted and cytokine signalling could induce Runx3 expression. However, once Runx3 expression was induced, Runx3 would not only repress ThPOK (Egawa and Littman, 2008; Setoguchi *et al*, 2008) and *Cd4* gene expression (Taniuchi *et al*, 2002; Sato *et al*, 2005), but Runx3 would also re-activate *Cd8* gene transcription (Taniuchi *et al*, 2002; Sato *et al*, 2005; Egawa *et al*, 2007) which, in 4in8 mice, would cause re-expression of *Cd8*-encoded CD4 proteins. CD4 re-expression on positively selected 4in8 thymocytes should restore the potential for renewed CD4-dependent TCR signalling, but these thymocytes continued their differentiation into cytotoxic-lineage T cells anyway. We interpret these findings as indicating that transient disruption of positive selection signalling that permits induction of Runx3 expression irreversibly commits thymocytes to the cytotoxic-lineage.

Cd8 gene expression is regulated by a combination of transcription factors and *cis* regulatory enhancers that combine with the *Cd8a* or *Cd8b* promoter to initiate and sustain CD8 transcription in developing thymocytes. The $E8_{II}$ and $E8_{III}$ enhancer elements have been shown to promote *Cd8* gene expression in immature DP thymocytes, whereas $E8_{II}$ and $E8_{I}$ enhancer elements promote *Cd8* gene expression in mature SP thymocytes (Ellmeier *et al*, 1999; Kioussis and Ellmeier, 2002; Feik *et al*, 2005). While $E8_{III}$ is only active in DP thymocytes and its activity is permanently terminated by TCR-mediated positive selection signals (Feik *et al*, 2005; Sarafova *et al*, 2005), $E8_{II}$ is active in both pre-selection DP thymocytes and mature SP T cells. Interestingly, we show here that TCR signalling of DP thymocytes terminated $E8_{II}$ activity, but only transiently, indicating that TCR-induced transient downregulation of *Cd8* gene expression during positive selection was mediated by downregulating the activity of specific *cis* regulatory elements. However, it should be appreciated that the $E8_{II}$-reporter transgene may not fully recapitulate the activity of the $E8_{II}$ enhancer in the endogenous *Cd8* gene locus.

In conclusion, the discovery of coreceptor gene imprinting provides a paradigm of how lineage fate determination occurs in developing thymocytes. Rather than determined by signalling differences between coreceptor proteins and/or MHC-specific TCR, the lineage factors that coreceptor-dependent thymocytes express and the lineage fate that coreceptor-dependent thymocytes adopt is determined by regulatory elements in *Cd4* and *Cd8* coreceptor gene loci that control the kinetics of coreceptor protein expression during positive selection. And it is the kinetics of coreceptor protein expression that result in thymocyte expression of different transcription factors that specify alternative lineage fates.

Materials and methods

Mice

C57BL/6 (B6), B10.A, $Cd4^{-/-}$, $B2m^{-/-}$, MHCII$^{-/-}$ ($IAb^{-/-}$), and bm12 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in our animal colony. Transgenic mice expressing AND TCR (Kaye *et al*, 1989), ThPOK(GFP) bacterial artificial chromosome reporter (Wang *et al*, 2008), and *Lck* proximal promoter-driven SOCS1 (Hanada *et al*, 2001) were maintained in our animal colony. Endogenous Runx3d(YFP) 'knock-in' reporter mice (Egawa and Littman, 2008) were a kind gift from Dan Littman (NYU). The $E8_{II}$-CD4 transgene consisted of the $E8_{II}$ *Cd8a* enhancer + *Cd8a* promoter driving CD4 cDNA and was constructed by re-engineering the TG-23 transgene (Ellmeier *et al*, 1998) so that it encoded murine CD4 rather than hCD2 proteins. The $E8_{I}$-CD4 and $E8_{III}$-CD4 transgenes have been previously described (Sarafova *et al*, 2005; Adoro *et al*, 2008). In this study, the $E8_{I}$-CD4, $E8_{II}$-CD4, and $E8_{III}$-CD4 transgenes were each expressed in CD4-deficient ($Cd4^{-/-}$) mice to exclude endogenous CD4 protein expression.

Generation of $Cd8a^{CD4}$ and 4in8 mice

The $Cd8a^{CD4}$ allele was generated using a targeting construct (Supplementary Figure S1), consisting of a CD4 cDNA downstream of a CD4 intronic splicing module (Sawada *et al*, 1994) and electroporated into murine 129 R1 ES cells. DNA from targeted ES cells was screened for recombination at the 5′-end by digestion with *Sac*I and southern blotting with a 5′-probe. Recombination at the 3′-end was confirmed by PCR (Supplementary Figure S1). Male $Cd8a^{T}$ knock-in mice were bred to EIIa-Cre transgenic female mice to induce germline deletion of the floxed Neo^{r} selection cassette (Lakso *et al*, 1996), giving rise to the $Cd8a^{CD4}$ gene. Knock-in mice were maintained as $Cd8a^{CD4/+}$ on a B6 background. $Cd8a^{CD4/+}$ mice were bred to $Cd4^{-/-}B2m^{-/-}$ mice to generate homozygous $Cd8a^{CD4/CD4}Cd4^{-/-}B2m^{-/-}$ mice, which we refer to as '4in8' mice. All mice were maintained under pathogen-free conditions in accordance with the US National Institutes of Health (NIH) guidelines.

Antibodies and flow cytometry

Monoclonal antibodies with the following specificities were obtained from BD Pharmingen: CD4 (GK1.5 and RM4.4), CD8α (53-6.7), CD69 (H1.2F3), TCRβH57–597), CD5 (53-7.3), CD24 (M1/69), CD44 (IM7), CD62L (MEL-14), CD154 (MR1), Vα2 (20.1), Vα3.2^{b,c} (RR3-16), Vα8.3 (B21.14), Vα11 (RR8-1), Vβ3 (KJ25), Vβ5.1,5.2 (MR9-4) and mouse Vβ TCR screening panel; granzyme B (16G6, eBioscience); CD8α (CT-CD8a, Caltag); and anti-Myc-FITC (9E10, Sigma). Cells were stained and analysed on a FACSVantage SE (Becton Dickinson) with four-decade logarithmic amplification. Dead cells were excluded by forward light scatter and propidium-iodide staining. Data were analysed using software designed by the Division of Computer Research and Technology, NIH.

Cell purification and sorting

Purified $CD4^+$ LN T cells were obtained by depletion of $CD8^+$ and Ig^+ cells; purified $CD8^+$ LN T cells were obtained by depletion $CD4^+$ and Ig^+ cells using Biomag beads (Qiagen), resulting in cell purities >95%. For cell sorting, single cell suspension of thymocytes or LN cells were stained with fluorochrome-conjugated antibodies and sorted on a FACSAria (Becton Dickinson). Immature pre-selection thymocytes used for *in vitro* signalling cultures were obtained by positive selection on peanut agglutinin (PNA)-coated plates (Suzuki *et al*, 1995). This protocol typically yielded >95% $TCR\beta^{lo}CD69^{-}CD24^{hi}$ immature thymocytes.

Functional assays

Mixed lymphocyte reactions were performed with 1×10^5 T cells labelled with 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) and 2×10^5 LPS-blasted splenocytes. For peptide stimulation, 10^5 CFSE-labelled AND T cells were cultured for 4 days with 2×10^5 I-E^k APC with or without 1 μM PCC.

For cytokine signalling, LN T cells (5×10^6 cells/ml) were cultured 16 h with recombinant mouse IL-7 (10 ng/ml; PeproTech), IL-4 (40 ng/ml; PeproTech), IL-6 (45 ng/ml; R&D Systems) or

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IL-15 (100 ng/ml; R&D Systems) as previously described (Park *et al*, 2007).

To signal thymocyte differentiation *in vitro*, purified immature pre-selection thymocytes were stimulated for 16 h with 0.3 ng/ml PMA and 0.3 μg/ml ionomycin and then placed into recovery cultures with medium alone for an additional 16 h. Where indicated, cells were pronase treated to remove preexisting surface coreceptors and placed in complete medium and assessed for surface coreceptor re-expression after 16–18 h (Suzuki *et al*, 1995).

To assay helper function, $2–5 \times 10^6$ B-cell-depleted LN T cells were stimulated for 6–8 h by immobilized anti-TCR/CD28 mAbs coated at 5 and 2 μg/ml, respectively, and then assessed for CD154 and CD69 expression (Maruo *et al*, 1997).

To assay cytotoxic function, LN T cells were assessed for their ability to lyse target cells in a re-directed cytotoxicity assay (Liu *et al*, 2002). Effector T cells were generated by 3-day stimulation with plate-bound anti-CD3/CD28 and then cultured for an additional 2 days in rhIL-2 (50 U/ml). ^{51}Cr-labelled L1210 (Fas-deficient) target cells were biotinylated and strepatividin coated and mixed with effector T cells in the presence or absence of 2.5 μg/ml biotinylated anti-CD3 for 4 h. Specific lysis (%) was calculated as: [([sample release]−[spontaneous release])/([maximum release]−[spontaneous release])] × 100. Specific lysis without anti-CD3 mAb was <5%.

For intracellular calcium flux, thymocytes were loaded with Indo-1 (Molecular Probes) for 30 min at 31 °C and then stained with biotinylated antibodies at the indicated concentrations; washed and pre-warmed to 37 °C for 2 min before being applied to the flow cytometer. Cells were signalled 30 s after being applied to the flow cytometer by avidin cross-linking.

For Th1 and Th2 differentiation experiments, purified CD4 or CD8 LN T cells were subjected to Th1 and Th2 differentiation cultures as previously described (Yamashita *et al*, 2000). Cells were stimulated for 2 days with plate-bound anti-TCR (5 μg/ml) and anti-CD28 (2 μg/ml) antibodies in the presence of recombinant human IL-2 (25 U/ml). For Th2 conditions, cultures were additionally supplemented with 10 ng/ml IL-4 (Peprotech) and 1 μg/ml anti-IFNγ (XMG1.2; BD Pharmingen) monoclonal antibody. For Th1 conditions, cultures were supplemented with 5 ng/ml IL-12 (p70; BD Pharmingen) and 1 μg/ml anti-IL-4 (11B11; BD Pharmingen) monoclonal antibody. After 2 days, stimulated cells were transferred to new plates and further cultured in Th1 or Th2 conditions without TCR/CD28 stimulation. At day 5, cells were harvested, washed, and stimulated with PMA and ionomycin for 4 h in the presence of GolgiStop™ containing monensin (BD Pharmingen). Stimulated cells were harvested, washed, and stained for intracellular expression of IFNγ and IL-4 as readout for Th1 and Th2 differentiation respectively.

Semi-quantitative PCR and real-time quantitative PCR

Total RNA was isolated from 1×10^6 T cells using the RNEasy protocol (Qiagen) and was reverse transcribed to cDNA by oligo(dT) priming with the SuperScript™ III First-Strand Synthesis System (Invitrogen). For qRT–PCR, cDNA was prepared using the RNEasy protocol (Qiagen) and assayed using the SYBR green detection system (Qiagen). Gene expression values was normalized to values of *Actb* (β-actin gene) in the same sample. Primers used of semi-quantitative RT–PCR: *Cd8b* (F: 5′-CCAAGATGGTCTTTGGGACAGG-3′; R: 5′-AAAGCAGGCAGCTTGAGCAAGG-3′); *Runx3d* (F: 5′-GTGAGCCTCGTTCATTCAT-3′; R: 5′-GGTCAGACCCACTTGGTTGG-3′); *Zbtb7b* (F: 5′-ACATGAGGACCCACACTGGTG-3′; R: 5′-CTTCCTCTTCCTCCTCCTCAG-3′); *Hprt* (F: 5′-GATACAGGCCAGACTTTGTTG-3′; R: 5′-GGTAGGCTGGCCTATAGGCT-3′); *Actb* (F: 5′-CAGGCATTGCTGACAGGATGC-3′; R: 5′-AAGGGTGTAAAACGCAGCTCAG-3′). Primers used for quantitative RT–PCR were *Zbtb7b* (F: 5′-ACATGAGGACCCACACTGGTG-3′; 5′-CTTCCTCTTCCTCCTCCTCAG-3′); *Cd8b* (F: 5′-CCAAGATGGTCTTTGGGACAGG-3′; R: 5′-AAAGCAGGCAGCTTGAGCAAGG-3′); 3. *Runx3d* (F: 5′-GCGACATGGCTTCCAACAGC-3′; R: 5′-CTTAGCGCGCCGCTGTTCTCGC-3′); *Actb* (F: 5′-GAGAGGGAAATCGTGCGTG A-3′; R: 5′-ACATCTGCTGGAAGGTGGAC-3′); *Gata3* (F: 5′-GTCCTCATCTCTTCACCTTCC-3′; R: 5′-GAGTCCGCAGGCATTGCAAAG-3′); *Tox* (F: 5′-CAGGACCCCTACTATTGCAAC-3′; R: 5′-GCAGGCCATTGTGATTCATGG-3′).

Western blotting

To determine CD4-bound Lck, 1×10^7 thymocytes were solubilized in 1% NP-40 and immunoprecipitated with anti-CD4 mAb and sepharose A beads. To determine TCR phosphorylated substrates, lysates from 1×10^7 thymocytes were immunoprecipitated with polyclonal rabbit anti-TCRζ antibody (serum 551) and sepharose A beads and immunoprecipitates were resolved by 10% SDS–PAGE.

Statistical analysis

Student's *t*-test with two-tailed distribution was used. *P*-values of $\leqslant 0.05$ were considered statistically significant.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: SA designed and performed the experiments, analysed the data, and wrote the manuscript; TM, FVL, JHP, XT, and MK did the experiments and analysed the data; BE and AA constructed targeting vector; AG and PL helped generate knock-in mice; MK, LW, and RB provided critical reagents; AS directed the study, analysed the data, and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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