TCF-1 and LEF-1 act upstream of Th-POK to promote the CD4+ T cell fate and interact with Runx3 to silence Cd4 in CD8+ T cells

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The transcription factors TCF-1 and LEF-1 are essential for early T cell development, but their roles beyond the CD4+CD8+ double-positive (DP) stage are unknown. By specific ablation of these factors in DP thymocytes, we demonstrated that deficiency in TCF-1 and LEF-1 diminished the output of CD4+ T cells and redirected CD4+ T cells to a CD8+ T cell fate. The role of TCF-1 and LEF-1 in the CD4-versus-CD8 lineage ‘choice’ was mediated in part by direct positive regulation of the transcription factor Th-POK. Furthermore, loss of TCF-1 and LEF-1 unexpectedly caused derepression of CD4 expression in T cells committed to the CD8+ lineage without affecting the expression of Runx transcription factors. Instead, TCF-1 physically interacted with Runx3 to cooperatively silence Cd4. Thus, TCF-1 and LEF-1 adopted distinct genetic ‘wiring’ to promote the CD4+ T cell fate and establish CD8+ T cell identity.

CD4+ and CD8+ T cells, the essential mediators of cellular immune responses, are produced in the thymus following sequential maturation stages. Hematopoietic progenitor cells first seed the thymus and then make ‘decisions’ for the specification of and commitment to the T cell lineage at the CD4–CD8– double-negative (DN) stage1,2. While recombination of the locus encoding the T cell antigen receptor β-chain (TCRβ) is completed at the CD25+CD44+ DN3 stage, rearrangements of the locus encoding TCRα occur after DN cells mature into CD4+CD8+ double-positive (DP) thymocytes, followed by negative and positive selection. The positively selected DP thymocytes first give rise to CD4+CD8lo intermediate cells, which then differentiate into major histocompatibility complex (MHC) class II-restricted CD4+ or MHC class I-restricted CD8+ single-positive (SP) T cells, an event known as the ‘CD4-versus-CD8 lineage choice’3.

The CD4-versus-CD8 lineage ‘choice’ is influenced by the timing, intensity and duration of signaling derived from the TCR and cytokines4. Various transcription factors intrinsically regulate this critical fate decision4,5. The factors c-Myb, GATA-3, Tox and Th-POK are specifically required for CD4+ T cell differentiation6–9, and combined mutations of the genes encoding Runx1 and Runx3 completely abrogate the production of CD8+ T cells with limited effects on the output of CD4+ T cells10,11. In terms of genetic interactions, c-Myb is required for the induction of GATA-3 expression by TCR signaling in DP thymocytes7. Upregulation of Th-POK expression is most evident in CD4+CD8lo intermediate cells12 and depends on both Tox and GATA-3 (refs. 6,9). Th-POK is needed to antagonize the activity and/or expression of Runx3 to promote commitment to the CD4+ T cell lineage11 and, conversely, Runx3-mediated repression of Th-POK expression is critical for the differentiation of CD8+ T cells10,12. Collectively, these findings indicate that the Th-POK–Runx3 axis seems to be a critical convergence point in determining CD4-versus-CD8 lineage fate.

Once the decision to become either CD4+ or CD8+ SP thymocytes is made, lineage-inappropriate genes must be silenced in the committed T cells to ensure their distinct identity and functional divergence. Thus far, silencing of genes encoding CD4+ T cell–specific molecules, such as the coreceptor CD4 itself and Th-POK, in CD8+ SP T cells is well characterized. Repression of Cd4 is mediated by a silencer sequence ~430 base pairs (bp) in length in its first intron13. Th-POK is encoded by Zbtb7b (called ‘Thpok’ here); repression of Thpok in CD8+ T cells is regulated by a silencer sequence ~560 bp in length upstream of Thpok exon 1a10,12. The silencers of both Cd4 and Thpok contain consensus binding motifs for Runx factors, and combined mutations of the genes encoding Runx1 and Runx3 result in derepression of Cd4 and Thpok in CD8+ T cells10,13.

TCF-1 and LEF-1 are members of the TCF-LEF family of transcription factors and have abundant expression in T cells14,15. TCF-1 is induced by activation of the Notch signaling pathway and is essential for Frontier Medical Sciences, Kyoto University, Kyoto, Japan. 8Department of Internal Medicine, Carver College of Medicine, University of Iowa, Iowa City, Iowa, USA. 9Present address: Department of Nephrology, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China. 10These authors contributed equally to this work. Correspondence should be addressed to H.-H.X. (hai-hui-xue@uiowa.edu).

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for the specification of hematopoietic progenitor cells to the T cell lineage16,17. TCF-1 and LEF-1 then act together to promote complete commitment to the T cell lineage, β-selection and the maturation of DN thymocytes to the DP stage18,19. In DN and preselection DP thymocytes, TCF-1 also restrains the expression of LEF-1, the transcription inhibitor Id2 and key components of the Notch signaling pathway to prevent malignant transformation18,20,21. However, because germline deletion of TCF-1 and LEF-1 causes a severe early T cell developmental block and embryonic death, respectively19,22, their roles beyond the DP stage are unknown. In this study, we overcame these obstacles by conditionally ablating both TCF-1 and LEF-1 in DP thymocytes through the use of Cre recombinase expressed from the promoter and enhancer of Cd4 (CD4-Cre). Loss of TCF-1 and LEF-1 specifically impaired the differentiation of CD4+ SP T cells from bipotent DP and CD4+CD8lo precursor cells and caused derepression of CD4 expression in committed CD8+ SP T cells. Our findings thus broaden the spectra of TCF-1- and LEF-1-mediated regulatory activities in late stages of T cell development and reveal new insights into cell-fate decision mechanisms and the establishment of cell identity.

RESULTS

The production of CD4+ T cells requires TCF-1 and LEF-1

To investigate the role of TCF-1 and LEF-1 in late stages of T cell development, we used CD4-Cre to conditionally inactivate both factors in DP thymocytes. Mice with loxp-flanked alleles encoding LEF-1 (Lef1flox) have already been established18. The gene encoding TCF-1 (Tcf7) was conditionally targeted by the International Knockout Mouse Consortium (project 37596); exon 4 of Tcf7 was flanked by two loxp sites, and deletion of this exon resulted in a nonsense frameshift mutation (Supplementary Fig. 1). Immunoblot analysis confirmed that deletion mediated by CD4-Cre was initiated in preselection DP thymocytes and was complete in post-selection DP cells. Both TCF-1 and LEF-1 are expressed in various isoforms in thymocytes due to differences in promoter use and alternative splicing, and all isoforms were effectively eliminated in post-selection DP cells (Fig. 1a).

Due to the requirements for TCF-1 in specification to the T cell lineage, β-selection and thymocyte survival, germline deletion of TCF-1 results in diminished thymic cellularity to <5% that of wild-type mice22. In contrast, CD4-Cre-mediated late deletion of TCF-1 (Tcf7−/−) or of both TCF-1 and LEF-1 (Tcf7−/−Lef1−/−) diminished the number of thymocytes only moderately, whereas thymic cellularity in Lef1−/−CD4-Cre (Lef1−/−) mice was similar to that in their TCF-1- and LEF-1-sufficient control littermates (details in the Online Methods section) (Fig. 1b). Although the frequency of TCRβhi subset was similar in thymuses from mice of each genotype, expression of the marker CD69 was lower on Tcf7−/−Lef1−/− TCRβhi thymocytes than on their counterparts on Lef1−/−, Tcf7−/− or control mice, as measured by mean fluorescence intensity (Fig. 1c and data not shown). The decreased CD69 expression was probably not the result of diminished TCR signaling, because TCR-dependent upregulation of Gata3 and Tox was not affected in Tcf7−/−Lef1−/− post-selection DP thymocytes (Supplementary Fig. 2). Downregulation of the expression of CD69 and CD24 marks the intrathymic maturation of positively selected TCRβhi thymocytes. We found that the maturation of CD24+CD69hi thymocytes to CD24−CD69− cells was not detectably perturbed in Lef1−/−, Tcf7−/− or Tcf7−/−Lef1−/− thymuses (Fig. 1d). The CD24+CD69− TCRβhi subset contains post-selection DP thymocytes and CD4+CD8hi intermediate cells, which are the immediate precursors of immature CD4+ or CD8+ SP thymocytes3. While T cell development was not apparently altered in Lef1−/− mice, we observed the accumulation of cells with a DP phenotype and a concomitant decrease in the abundance of both CD4+ SP thymocytes and CD8+ SP thymocytes in Tcf7−/− and Tcf7−/−Lef1−/− thymuses (Fig. 1e). The CD24−CD69− TCRβhi subset contains mature SP thymocytes only11,
and deletion of TCF-1 alone or together with LEF-1 progressively diminished the frequency of CD4+ SP T cells (Fig. 1f), which suggested a requirement for TCF-1 and LEF-1 in the effective production of CD4+ thymocytes.

**TCF-1 and LEF-1 repress CD4 expression in CD8+ T cells**

A fraction of mature Tcf7−/− CD8+ SP thymocytes showed increased expression of CD4, and this fraction was substantially larger for Tcf7−/− Lef1−/− CD8+ T cells (Fig. 1f). By separating Tcf7−/− Lef1−/− TCRβhi CD8+ cells into the CD8+CD4− subset and the CD8+CD4+ subset (called ‘CD8*4’ here to distinguish these cells from true, immature DP cells), we found that both subsets expressed the monomorphic coreceptor CD8β (Fig. 2a). Although this was not a focus of our study, we noted that the expression of both CD8α and CD8β was moderately lower in Tcf7−/− Lef1−/− TCRβhi CD8+ T cells than in their control counterparts (Fig. 2a). We next measured the expression of genes that are characteristic of CD4+ or CD8+ SP T cells. Runx3 is expressed in both cell types, but a distal promoter of Runx3 is used exclusively in CD8+ T cells, which generates a Runx3d transcript. In contrast, Thpok is expressed only in CD4+ T cells. The CD4−CD8+ and CD8*4 subsets of Tcf7−/− Lef1−/− thymocytes both expressed Runx3d but not Thpok (Fig. 2b). In addition, there was higher expression of total Runx3 and Prf1 (which encodes perforin) in naive CD8*4 SP T cells than in CD4+ SP T cells, and this trend was preserved in the CD8*4CD4− and CD8*4 subsets of Tcf7−/− Lef1−/− thymocytes (Fig. 2b). These data suggested that the CD8*4 cells from Tcf7−/− Lef1−/− mice were true cytotoxic CD8*4 T cells with derepressed CD4 expression, similar to Runx3- or Runx3d-deficient CD8+ T cells1,13.

To further substantiate that point, we crossed Tcf7−/− Lef1−/− mice and control mice onto an H2-Ab1−/− background, in which CD4+ T cells are much less abundant due to the lack of MHC class II I-A and I-E molecules24. The MHC class I–selected T cells showed derepression of CD4 expression in the absence of TCF-1 and LEF-1 (Fig. 2c), which formally excluded the possibility that the CD8*4 T cells were CD4+ T cells with improper expression of CD8. These findings suggested a role for TCF-1 and LEF-1 in the silencing of CD4 in CD8+ T cells.

**TCF-1 and LEF-1 are critical for the CD4+ T cell fate**

Due to the derepression of CD4 expression in Tcf7−/− Lef1−/− CD4*4 SP thymocytes, true DP cells and CD8*4 cells could not be distinguished phenotypically in the immature CD24*CD69+ TCRβhi compartment. To accurately measure the output of CD4+ or CD8*4 SP T cells, we therefore focused on mature CD24*CD69+ TCRβhi thymocyte populations, which do not include DP cells in wild-type mice. Loss of TCF-1 alone caused a decrease of 40% in the number of mature CD4+ SP thymocytes, and deletion of both TCF-1 and LEF-1 caused a decrease of >80% (Fig. 3a). Whereas TCF-1 deficiency did not have a significant effect on the number of mature CD8*4 SP thymocytes (counted as the sum of CD8*4CD4− and CD8*4CD4+ cells), loss of both TCF-1 and LEF-1 substantially increased the number of CD8*4 SP T cells (Fig. 3a). The ratio of mature CD4+ SP cells to CD8*4 SP cells is approximately 2:1 in wild-type mice, but this ratio was diminished to 1:1 in Tcf7−/− mice and was reversed to about 0.1:1 in Tcf7−/− Lef1−/− mice (Fig. 3b). The same phenotypic defects, including a lower frequency and number of CD4+ SP T cells and a decreased ratio of CD4+ to CD8*4 T cells, persisted in the periphery (Supplementary Fig. 3a–c).

Germline deletion of TCF-1 diminishes thymic cellularity by >95%, due in part to a critical requirement for TCF-1 for the survival of early thymocytes25. By measuring active caspase-3 and caspase-7, we confirmed that early deletion of Tcf7 caused the activation of caspase in ~35% of post-selection TCRβhi thymocytes (Supplementary Fig. 3d). However, the increase in caspase activation in TCRβhi thymocytes from Tcf7−/− or Tcf7−/− Lef1−/− mice was rather moderate (Supplementary Fig. 3e), which indicated that CD4-Cre–mediated late deletion of Tcf7 and Lef1 greatly alleviated the dependence of post-selection thymocytes on TCF-1 and LEF-1 for survival. Notably, caspase activation was similar in mature CD4+ or CD8*4 SP thymocytes in Tcf7−/− and Tcf7−/− Lef1−/− mice (Supplementary Fig. 3f). These data suggested that TCF-1 and LEF-1 critically regulated the CD4+ T cell lineage choice rather than ‘preferentially’ promoting the survival of CD4+ T cells. In addition, the residual CD4+ T cells in H2-Ab1−/− mice, which may have been selected on the H-2O MHC class II molecule26, were completely abolished by loss of TCF-1 and LEF-1 (Fig. 2c), which lent additional support for the idea of an essential
Figure 3  Deficiency in TCF-1 and LEF-1 redirects CD4+ T cells to the CD8+ T cell lineage. (a) Quantification of mature CD4+ or CD8+ SP thymocytes in control, Left−/−, Tcf7−/− and Tcf7−/−Lef1−/− mice (n ≥ 6 per genotype). (b) Ratio of mature CD4+ cells to CD8+ cells (CD4/CD8), calculated from the data in a. Each symbol represents an individual mouse; small horizontal lines indicate the mean. (c) Distribution of donor-derived (CD45.2+) mature CD69−CD24−TCRhi thymocytes into the CD4+ or CD8+ lineage 6 weeks after transplantation of BM cells from donor Tcf7−/− or Tcf7−/−Lef1−/− mice or their control littermates into lethally irradiated congenic (CD45.1+) B2m−/− host mice. Numbers adjacent to outlined areas indicate percent CD4+ cells (top left) or CD8+ cells (bottom right). (d) Quantification of mature CD4+ or CD8+ thymocytes in the B2m−/− chimeras in e (n ≥ 14 recipients for each genotype of donor cells). (e) Distribution of mature CD4+ thymocytes into the CD4+ or CD8+ lineage in OT-II Tcf7−/− and Tcf7−/−Lef1−/− mice or their control littermates (n ≥ 5 per genotype), assessed after gating on the Vα2TCRhi subset. (f) Frequency of mature CD4+ or CD8+ OT-II thymocytes in e. * P < 0.05, ** P < 0.01 and *** P < 0.001 (t-test). Data are representative of six or more experiments (a,b), four independent experiments (c,d) or five experiments (e,f); mean and s.d. in a,d,f.

role for TCF-1 and LEF-1 in promoting the differentiation of DP thymocytes into the CD4+ T cell lineage.

Deficiency in Th-POK or GATA-3 results in redirection of CD4+ T cells to the CD8+ lineage6.8. In Tcf7−/−Lef1−/− mice, the decrease in the abundance of CD4+ SP thymocytes was accompanied by an increase in CD8+ SP cells (Fig. 3a), indicative of lineage redirection. To further assess this, we transplanted CD45.2+ bone marrow (BM) cells from Tcf7−/− or Tcf7−/−Lef1−/− mice (we did not use Left−/− cells because loss of LEF-1 alone showed little effect) or their control littermates into lethally irradiated congenic CD45.1+ recipient mice deficient in β2-microglobulin (B2m−/− mice). B2m−/− mice are defective in the expression of MHC class I and thus have very few CD8+ SP T cells27. In B2m−/− hosts, mature TCRβ thymocytes derived from BM cells of the control littermates noted above were predominantly CD4+ (Fig. 3c,d). In contrast, Tcf7−/− BM cells gave rise to substantial numbers of mature CD8+ thymocytes, and mature thymocytes generated from Tcf7−/−Lef1−/− BM cells were predominantly CD8+ (Fig. 3c,d). These data indicated that MHC class II–selected thymocytes underwent a change in fate from CD4+ T cells to CD8+ T cells in the absence of TCF-1 alone or both TCF-1 and LEF-1. Consistent with the finding of an essential role for TCF-1 and LEF-1 in the silencing of TCRβ expression in the CD8+ compartment (Fig. 3c,d). In both Tcf7−/− and Tcf7−/−Lef1−/− mice, we also observed derepression of CD4 expression in the redirected OT-II CD8+ T cells (Fig. 3e). Of note, CD4-Cre–mediated deletion of TCF-1 alone or both TCF-1 and LEF-1 caused a greater decrease in total thymic cellularity in the presence of the OT-II transgene (Supplementary Fig. 5a) than in its absence (Fig. 1b). This was probably due to altered timing of excision of the target gene(s) by CD4-Cre because of the OT-II transgene, as we observed early deletion of Tcf7 and Left in DN thymocytes from OT-II Tcf7−/−Lef1−/− mice (Supplementary Fig. 5b). Nonetheless, mature OT-II CD8+ thymocytes were more abundant than CD4+ SP thymocytes after deletion of TCF-1 alone or both TCF-1 and LEF-1 (Supplementary Fig. 5c). Collectively, loss of TCF-1 and LEF-1 resulted in a fate change...
of MHC class II–selected cells to the CD8+ lineage whether the post-selection DP thymocytes expressed polyclonal TCRs or a fixed MHC class II–restricted TCR. These findings revealed an essential role for TCF-1 and LEF-1 in directing bipotent precursor cells to the CD4+ T cell lineage.

TCF-1 regulates balanced expression of *Thpok* and *Runx3d*

Several transcriptional factors have been characterized as being intrinsic regulators of the CD4+-versus-CD8+ fate decision. Among these, c-Myb, GATA-3, Tox and Th-POK direct post-selection DP cells to the CD4+ T cell lineage, and Runx factors ensure CD8+ T cell differentiation. We thus investigated if TCF-1 and LEF-1 regulate these key factors involved in lineage choice. Because double deficiency in TCF-1 and LEF-1 resulted in strong derepression of CD4 expression in CD8+ SP T cells (Fig. 2), the resulting CD8+ 4 cells could not be adequately separated from actual post-selection DP thymocytes in Tcf7−/− Lef1−/− mice. To avoid misinterpretation of the data, we focused our gene-expression analysis on Tcf7−/− thymocytes.

To discern kinetic changes in gene expression at distinct stages before complete lineage commitment, we purified pre-selection (TCRβ−/−) DP cells, post-selection (TCRβ+β) DP cells and CD4+CD8α intermediate cells by cell sorting. Similar to the results obtained by immunoblot analysis (Fig. 1a), CD4-Cre–mediated deletion of Tcf7 was more complete at the post-selection DP stage and beyond (Fig. 4a). While Tcf7 expression was relatively constant throughout these stages, Lef1 exhibited dynamic changes in expression similar to those of Gata3 and Tox, as it was upregulated by positive selection signals and had a sustained high expression in CD4+CD8α thymocytes.9,10 *Thpok* expression was induced at the post-selection stage and was induced more potently in CD4+CD8α cells (Fig. 4b–d). Deletion of TCF-1 did not affect kinetic changes in the expression of Gata3, Tox and Myb (Fig. 4c–e) but substantially diminished the expression of *Thpok* in both post-selection DP thymocytes and CD4+CD8α thymocytes (Fig. 4f). TCF-1 deficiency did not significantly alter the expression of Runx1 or total Runx3 (transcribed from both the distal promoter and proximal promoter) (Fig. 4g–h). Runx3 protein in CD8+ SP T cells is produced exclusively from the Runx3d transcript and in fact transcription from the Runx3 distal promoter is initiated at the post-selection DP stage.11,12 Notably, specific deletion of Runx3d and complete ablation of Runx3 have similar effects on the differentiation of DP thymocytes into the CD8+ lineage and silencing of Cda in CD8+ T cells11,12. Thus, *Runx3d* expression is directly linked to the activity of Runx3 in lineage choice. Detection of *Runx3d* transcripts by quantitative RT-PCR with primers specifically complementary to cDNA transcribed from the Runx3 distal promoter revealed that TCF-1 deficiency resulted in increased expression of *Runx3d* in both post-selection DP cells and CD4+CD8α cells (Fig. 4i). These gene-expression analyses collectively suggested that TCF-1 and LEF-1 affected the balanced expression of *Thpok* and *Runx3d* to regulate the CD4-versus-CD8 fate decision.

TCF-1 and LEF-1 act upstream of Th-POK

Because Th-POK and Runx3d mutually antagonize each other’s expression and/or activity,12,28 we next investigated which is the main factor that acts downstream of TCF-1 and LEF-1. We crossed Tcf7−/− or Tcf7−/− Lef1−/− mice to a mouse strain that expresses a transgene encoding Th-POK. This transgene is under the control of human C2B2–based regulatory elements and is expressed in DP and SP thymocytes.29 Consistent with published reports,6,29 such ectopic expression of Th-POK directed all post-selection DP thymocytes to the CD4+ lineage regardless of MHC restrictions. In Tcf7−/− mice expressing this transgene, overexpression of Th-POK suppressed the differentiation of CD8+ T cells and increased the frequency of CD4+ T cells among immature and mature TCRβ+ thymocytes (Fig. 5a). Notably, those mice had numbers of mature CD4+ SP T cells similar to those of their TCF-1-sufficient control littermates that did not have the transgene (Fig. 5b). These data suggested that ectopic expression of Th-POK was sufficient to ‘rescue’ the CD4+ T cell–differentiation defects caused by loss of TCF-1.

Although Tcf7−/− Lef1−/− mice had increased output of CD8+ T cells at the expense of CD4+ T cells, CD8+ T cell differentiation was suppressed in Tcf7−/− Lef1−/− mice expressing the transgene encoding Th-POK (Fig. 5a,b). Consequently, mature TCRβ+ thymocytes in those mice were almost exclusively CD4+, and the frequency of CD4+ SP thymocytes in the immature TCRβ+ subset of those mice (Fig. 5a) was greater than that in Tcf7−/− Lef1−/− mice without the transgene (Fig. 1e,f). The number of mature CD4+ SP thymocytes in Tcf7−/− Lef1−/− mice expressing the transgene encoding Th-POK was about 50% greater than that of Tcf7−/− Lef1−/− mice without the transgene had, but this number remained substantially lower than that of their TCF-1- and LEF-1-sufficient control littermates without the transgene (Fig. 5b). These observations suggested that double deficiency in TCF-1 and LEF-1 caused additional alterations beyond those that resulted from loss of TCF-1 alone and, as a consequence, the defective CD4+ T cell differentiation was not sufficiently reversed by overexpression of Th-POK. In line with that, the combination of overexpression of Th-POK and double deficiency in TCF-1 and LEF-1 may have had more complex effects on late T cell development, causing...
Figure 5  Ectopic expression of Th-POK rectifies the defects in CD4⁺ T cell differentiation caused by loss of TCF-1. (a) Distribution of immature and mature TCRβ⁺ thymocytes into the CD4⁺ or CD8⁺ lineage in control, Tcf7⁻/⁻ and Tcf7⁻/⁻Lef1⁻/⁻ mice expressing a transgene encoding Th-POK (Th-POK TG). Numbers adjacent to outlined areas indicate percent CD4⁺ SP, CD4⁺CD8⁺ DP or CD8⁺ SP cells (clockwise from top left). (b) Quantification of total thymocytes (top) and mature CD4⁺ and CD8⁺ SP thymocytes (middle and bottom) in control, Tcf7⁻/⁻ and Tcf7⁻/⁻Lef1⁻/⁻ mice (n ≥ 6 per genotype) with (+) or without (−) expression of the transgene encoding Th-POK. (c) Distribution of CD45.2⁺ mature TCRβ⁺ thymocytes into the CD4⁺ or CD8⁺ lineage 6 weeks after transplantation of BM cells from donor CD45.2⁺ mice (genotypes as in a) into irradiated CD45.1⁺ B2m⁻/− recipients. Numbers adjacent to outlined areas indicate percent CD4⁺ cells (top left) or CD8⁺ cells (bottom right). (d) Expression of Thpok, Runx3 and Runx3d in post-selection DP and CD4⁺CD8⁺ intermediate thymocytes from control or Tcf7⁻/− mice with or without expression of the transgene encoding Th-POK; results were normalized to Hprt expression. NS, not significant; *P < 0.05, **P < 0.01 and ***P < 0.001 (t-Test). Data are representative of three or more experiments (a,c,d) or are from five experiments (b; mean and s.d. in b,d).

We next examined the effect of ectopic expression of Th-POK on the redirection of CD4⁺ T cells to the CD8⁺ lineage caused by loss of TCF-1 alone or of both TCF-1 and LEF-1. We transplanted BM cells from Tcf7⁻/⁻ or Tcf7⁻/⁻Lef1⁻/⁻ mice expressing the transgene encoding Th-POK into CD45.1⁺ B2m⁻/− mice. In the chimeric hosts, the lineage redirection noted above (Fig. 3c) was completely blocked by expression of the transgene encoding Th-POK in mature TCRβ⁺ thymocytes (Fig. 5c). In line with that observation, ectopic expression of Th-POK diminished the expression of Runx3 and Runx3d in TCF-1-sufficient post-selection DP thymocytes and CD4⁺CD8⁺ thymocytes and, more notably, it prevented upregulation of Runx3d expression in Tcf7⁻/− cells (Fig. 5d).

These data collectively suggested that Th-POK acted downstream of TCF-1 and LEF-1 in regulating the CD4⁺ versus CD8⁺ lineage choice.

We then investigated whether TCF-1 directly regulates Thpok expression. Published studies have shown that the region flanking the first coding exon of Thpok, from −17 kilobases (kb) upstream to +1 kb downstream of the transcription start site, contains all the cis elements required for dynamic expression of Thpok during thymocyte development.12. In this region we found seven conserved consensus binding sequences for TCF-LEF ([(T/A)CAAAG, where ‘(T/A)’ means either thymidine or adenosine] between exons 1a and 2 of Thpok (motifs A–G, Fig. 6a). We used an antibody to TCF-1 (anti-TCF-1)10 or, as a control antibody, immunoglobulin G (IgG), for chromatin immunoprecipitation (ChIP) in sorted post-selection DP thymocytes and CD4⁺CD8⁺ thymocytes. We observed enrichment for the binding of TCF-1 to gene segments of Lef1 and Axin2 (Fig. 6b), two known targets of TCF-1. Among the seven conserved TCF-LEF motifs, TCF-1 bound specifically to motif D (Fig. 6b). The binding of TCF-1 to these genomic locations was abrogated in sorted post-selection DP thymocytes and CD4⁺CD8⁺ thymocytes from Tcf7⁻/− mice (Fig. 6b), indicative of binding specificity. Notably, motif D is located in an already defined ‘general T lymphoid element’ (GTE) that contributes to the positive regulation of Thpok in T cells.12. A published study analyzed TCF-1 in whole thymocytes by ChIP followed by deep sequencing (ChIP-Seq)13. Further analysis of the those data identified a strong TCF-1-binding peak at the Thpok GTE (Supplementary Fig. 6a), consistent with our scanning of the Thpok regulatory region by ChIP followed by PCR (Fig. 6a,b).

We found that the 473-bp GTE in Thpok contains two sets of highly conserved CAAAG motifs (Supplementary Fig. 7a,b). To investigate the contribution of these potential TCF-1-binding sites to the enhancer activity of GTE, we cloned the wild-type GTE either upstream or downstream of a luciferase reporter driven by an SV40 promoter. We then mutated both ‘CAAAG’ motifs in the GTE to ‘ACCCCT’ to generate mutant reporter constructs. Regardless of the location of insertion, inclusion of the wild-type GTE increased the reporter activity over that driven by the SV40 promoter alone and, notably, mutation of both TCF-1-binding sites almost completely abrogated the increase (Fig. 6c). We observed this in the EL4 mouse thymoma cell line (Fig. 6c) as well as in 293T human embryonic kidney cell lines (Supplementary Fig. 7c). Collectively, these findings supported the idea that TCF-1 acted directly upstream of Th-POK in directing bipotent DP or CD4⁺CD8⁺ precursor cells to the CD4⁺ T cell lineage.

TCF-1 and LEF-1 do not require Runx3 for lineage choice

We showed above that TCF-1 positively regulated Thpok expression, and Th-POK in turn repressed Runx3d expression. A parallel
mechanism could be that TCF-1 directly represses the expression of Runx3d and Runx3d then negatively regulates Thpok. To investigate this, we ablated total Runx3 expression by crossing Runx3−/−CD4-Cre (Runx3−/−) mice to the Tcf7−/− and Tcf7−/−Leif−/− strains noted above. Runx3−/− mice have moderately fewer CD8+ SP T cells in the thymuses and periphery23. However, Tcf7−/−Runx3−/− mice had a lower frequency and number of CD4+ T cells among both immature TCRβα thymocytes and mature TCRββ thymocytes than did Runx3−/− mice (Fig. 7a,b). Moreover, Tcf7−/−Leif−/−Runx3−/− mice exhibited a greater loss of mature CD4+ SP thymocytes, with CD8+ T cells remaining dominantly abundant (Fig. 7a,b), similar to Tcf7−/−Leif−/− mice (Figs. 1c,f and 3a). Thus, Runx3 deficiency failed to rectify the defective CD4+ lineage choice noted in Tcf7−/− or Tcf7−/−Leif−/− mice. In line with that, we found that Thpok expression in post-selection DP and CD4+CD8β thymocytes from Runx3−/− mice was similar to that of their TCF-1- and LEF-1-sufficient control littermates. Notably, Tcf7−/−Runx3−/− post-selection DP and CD4+CD8β thymocytes had lower expression of Thpok transcripts, similar to that of Tcf7−/− cells (Fig. 7c).

Overexpression of Runx3 increases the output of CD8+ T cells, but whether this is a result of lineage redirection remains controversial32. To determine if the increased frequency and number of CD8+ T cells in Tcf7−/−Leif−/− mice resulted from aberrant upregulation of Runx3d expression and ensuing lineage redirection, we generated B2m−/− chimeras with donor BM cells from Runx3−/−, Tcf7−/−Runx3−/− or Tcf7−/−Leif−/−Runx3−/− mice. As expected, mature TCRββ thymocytes derived from Runx3−/− BM cells were predominantly CD4+ in the B2m−/− recipients (Fig. 7d). However, mature TCRββ thymocytes derived from Tcf7−/−Runx3−/− or Tcf7−/−Leif−/−Runx3−/− BM cells exhibited redirection from the CD4+ T cell lineage to the CD8+ T cell lineage (Fig. 7d), similar to those from Tcf7−/− or Tcf7−/−Leif−/−
Figure 8 TCF-LEF and Runx factors act together in silencing Cd4 in CD8+ T cells. (a) Occupancy of Axin2 (left) and Cd4 (right) by TCF-1 in splenic CD8+ T cells, analyzed by ChIP-Seq with anti-TCF-1 or IgG, followed by uploading of ChIP-Seq track 'wiggle' files to the UCSC genome browser (top two rows), and enrichment for TCF-1-binding peaks, obtained by normalization of signals obtained by ChIP-Seq with anti-TCF-1 to those obtained by ChIP-Seq with IgG (bottom row). Bottom, gene structures; arrows indicate transcription orientation. (b) ChIP analysis of the binding of TCF-1 to the Cd4 silencer (left) or to Gapdh (negative control) or Lef1 (positive control) (right) in wild-type or Tcf7l1−/− CD8+ or CD4+ SP thymocytes; results obtained with anti-TCF-1 were normalized to those obtained with IgG (control) and are presented relative to enrichment at the Gapdh locus, set as 1. (c,d) Identification of the TCF-LEF-binding motif (c) and the Runx-binding motif (d) in the stringent TCF-1-binding peaks in a. (e) Distribution of TCF-LEF- and Runx-binding motifs in the stringent TCF-1-binding peaks in a. (f) Immunoassay of lysates of 293T cells overexpressing Myc-tagged Runx3 (Myc–Runx3) and Flag-tagged full-length TCF-1 (Flag–TCF-1), or the empty retroviral expression vector MigR1, assessed by immunoprecipitation (IP) with anti-Flag or IgG (control), followed by immunoblot analysis with anti-Myc; Input (bottom), lysate without immunoprecipitation (throughout). (g) Immunoassay of lysates of 293T cells overexpressing Myc-tagged Runx3 or transfected with empty vector expressing the Myc tag (pCMV-Myc), together with Flag-tagged full-length TCF-1 (Flag–p45 TCF-1) or the p33 TCF-1 isoform (Flag–p33 TCF-1), assessed by immunoprecipitation with anti-Myc, followed by immunoblot analysis with anti-Flag. (h) Immunoassay of lysates of 293T cells overexpressing Runx3Δ or mutant Runx3 lacking the WWRPY motif (Runx3ΔΔC), or empty vector (MigR1), together with Flag-tagged full-length TCF-1, assessed by immunoprecipitation with anti-Runx or IgG, followed by immunoblot analysis with anti-Flag. (i) Flow cytometry of control, Tcf7l1−/−, Tcf7l1−/−/Lef1−/−, Runx3Δ−/−, Tcf7l1−/−/Runx3Δ−/−, or Tcf7l1−/−/Lef1−/−/Runx3Δ−/− mature TCRhi T cells. Numbers adjacent to outlined areas indicate percent CD8+ or CD8+CD4− cells (%). (j) Immunoassay of lysates of 293T cells overexpressing Myc-tagged Runx3 (Myc–Runx3) or transfected with empty vector expressing the Myc tag (pCMV-Myc), together with Flag-tagged full-length TCF-1 (Flag–TCF-1) or the p33 TCF-1 isoform (Flag–p33 TCF-1), assessed by immunoprecipitation with anti-Myc, followed by immunoblot analysis with anti-Flag. (a) Flow cytometry of control, Tcf7l1−/−, Tcf7l1−/−/Lef1−/−, Runx3Δ−/−, Tcf7l1−/−/Runx3Δ−/−, or Tcf7l1−/−/Lef1−/−/Runx3Δ−/− mature TCRhi T cells. Numbers adjacent to outlined areas indicate percent CD8+ cells (top right) or CD8+CD4− cells (bottom right) in the CD8+ population (to avoid the influence of variation in the frequency of CD4+ cells). (j) Frequency of the CD8+4 subset for the cells in (i) (n ≥ 5 mice per genotype). *P < 0.05, **P < 0.01 and ***P < 0.001 (t-test). Data are from one experiment (a,c,d), are representative of at least three independent experiments (f-h) or are pooled from three or more independent experiments (b, j).

BM cells (Fig. 3c). Therefore, regardless of whether TCF-1 and LEF-1 directly or indirectly suppress Runx3 expression, Runx3 proteins were not essential for TCF-1- and LEF-1-mediated regulation of the CD4 versus CD8 lineage choice.

TCF-LEF and Runx3 factors act together in silencing Cd4

Detailed mapping of cis elements in the Cd4 silencer has revealed that several sites, in addition to the Runx-binding motifs, contribute to the stable repression of Cd4 in CD8+ T cells13,33. However, the identity of the factors that bind to these additional sites has remained unknown. To better understand the role of TCF-1 and LEF-1 in this process, we analyzed genome-wide occupancy by TCF-1 in naive splenic CD8+ T cells by ChIP-Seq with anti-TCF-1 (ref. 30) or the control antibody IgG and used the MACS (‘model-based analysis for ChIP-Seq’) algorithm84 to identify TCF-1-binding peaks. By the stringent criteria of enrichment of fourfold or more, a P value of <10−5 and a false-discovery rate of <5%, we identified 2,827 stringent TCF-1-binding peaks with high confidence. Through the use of the more permissive criterion of a P value of <10−3, we located additional 6,577 ‘permissive’ TCF-1-binding peaks (Supplementary Fig. 8a). Genomic distribution analysis of all TCF-1-binding peaks revealed that only 6% were located in the promoter regions (within the region −5 kb upstream to +1 kb downstream of the transcription start site) of known genes in the RefSeq reference sequence database of the National Center for Biotechnology Information (Supplementary Fig. 8b). Two histone-modification marks, the activating H3K4me3 mark and repressive H3K27me3 mark, have already been mapped by ChIP-Seq in human naive CD8+ T cells15. Peak-overlap analysis showed that 15% of the TCF-1-binding peaks in the
promoter regions overlapped H3K4me3 marks, whereas the overlap with H3K27me3 marks was lower (Supplementary Fig. 8c). Among the stringent TCF-1-binding peaks, we confirmed the direct association of TCF-1 with its known target genes, including Axin2 and Left1 (Fig. 8a and data not shown). We found one TCF-1-binding peak in the first intron of Cd4 at a location corresponding to the Cd4 silencer (Fig. 8a). Analysis of TCF-1 by ChiP-Seq in whole thymocytes31 confirmed binding of TCF-1 to the same location in Cd4 (Supplementary Fig. 6b). A published study has also reported ChiP-Seq analysis of Runx3 in CD8+ T cells and natural killer cells36. Collective analysis of these three sets of ChiP-Seq data revealed that binding of TCF-1 at intron 1 of Cd4 perfectly aligned with the Runx3-binding peak (Supplementary Fig. 6b), which suggested co-occupancy of TCF-1 and Runx3 at the silencer indeed contains a perfect TCF-LEF-binding motif (ACAAAG) in its 3′ terminus, with two known Runx-binding motifs in its 5′ half. We confirmed direct binding of TCF-1 to the Cd4 silencer in wild-type CD8+ T cells; this binding was abrogated in Tcf7−/− CD8+ T cells (Fig. 8b). The Cd4 silencer was not occupied by TCF-1 in CD4+ T cells (Fig. 8b), which suggested lineage specificity. Consistent with that observation, the binding of Runx3 to the Cd4 silencer is specific to CD8+ T cells and is not observed in natural killer cells36.

De novo motif-discovery analysis of the stringent TCF-1-binding peaks identified the known consensus TCF-LEF-binding motif in 52.5% of the peaks and the Runx-binding motif in 54.3% of the peaks (Fig. 8c,d). Overall, 908 peaks (32% of the total) contained both TCF-LEF- and Runx-binding motifs (Fig. 8c). Motif analysis of the 6,577 weak TCF-1-binding peaks revealed a similar trend, with TCF-LEF- and Runx-binding motifs present in 37.3% and 41.4% of the peaks, respectively, and 1,257 peaks containing both motifs (Supplementary Fig. 8d–f). By applying our stringent peak-calling setting to the ChiP-Seq data obtained for Runx3 in CD8+ T cells36, we identified 4,785 Runx3-binding peaks, and 1,270 of those overlapped with strong TCF-1-binding peaks (data not shown). These high-throughput data suggested that TCF-LEF and Runx factors may have a broadly cooperative role in gene regulation. It has been shown that Runx3 interacts with TCF-4 and attenuates signaling via TCF-4–β-catenin during intestinal tumorigenesis37. To further substantiate this point, we overexpressed Myc-tagged Runx3 with the Flag-tagged full-length p45 TCF-1 isoform in 293T cells and immunoprecipitated cell lysates with an anti-Flag or the control antibody IgG. Immunoblot analysis with anti-Myc revealed that TCF-1 immunoprecipitated together with Runx3 (Fig. 8f). In the reciprocal experiment, we detected Flag-tagged p45 TCF-1 after immunoprecipitation with anti-Myc (Fig. 8g). The p33 isoform of TCF-1, which is truncated at the amino terminus and lacks the β-catenin-binding domain, also immunoprecipitated together with Runx3 (Fig. 8g). Runx3 recruits the corepressors Groucho-TLE through its most carboxy-terminal five amino acids, Val-Trp-Pro-Arg-Tyr (the ‘VWPRY’ motif)38. Specific deletion of this sequence in the mouse germline causes derepression of CD4 expression in CD8+ T cells38, similar to complete truncation mutant did immunoprecipitate together with TCF-1 (Fig. 8h), which suggested that the direct physical interaction between TCF-1 and Runx3 occurred independently of Groucho-TLE.

To further delineate if TCF-LEF and Runx factors act in synergy in vivo, we examined derepression of CD4 expression in CD8+ T cells. In Tcf7−/− or Runx3−/− mice, a small portion of mature CD8+ thymocytes showed derepression of CD4 expression; however, in Tcf7−/−Runx3−/− mice, a much larger portion of CD8+ T cells expressed CD4 (Fig. 8i,j). Tcf7−/−Runx3−/− mice showed stronger derepression of CD4 expression in CD8+ T cells than did Tcf7−/− mice (Fig. 8i) because of the functional redundancy between TCF-1 and LEF-1 (Fig. 2), while Tcf7−/−Runx1−/− mice showed a further increase in the frequency of CD8+4 cells among mature TCRβ+ CD8+ thymocytes, with cells in the CD8+CD4− gate showing a strong shift toward increased CD4 expression (Fig. 8i), analogous to what is observed in Runx3−/−Runx1−/− mice (which express the Runx1Δ446 mutant that lacks the VWPRY motif)10,39. These observations demonstrated functional synergy between TCF-LEF and Runx factors in achieving stable silencing of Cd4 in CD8+ T cells.

**DISCUSSION**

TCF-1 and LEF-1 have well-documented roles in early T cell development. By conditional targeting of both factors, our studies have revealed their roles in late developmental stages, CD4+ versus CD8+ lineage choice and establishing CD8+ T cell identity. Lineage specification and commitment involve the activation of lineage-appropriate genes and inactivation of lineage-inappropriate genes40. Before lineage commitment, the DP precursor cells are probably biased toward CD4+ specification3, because the post-selection DP thymocytes lacking both Th-Pok and the Runx complex adopt a CD4+ T cell fate11. Adding to the factors known to promote lineage specification, such as c-Myb, Tox and GATA-3, we have now identified TCF-1 and LEF-1 as independent factors that promoted specification to the CD4+ lineage. Whereas expression of c-Myb was downregulated by positive selection signals, expression of Tox and GATA-3 was upregulated by such signals. In contrast, expression of TCF-1 and LEF-1 was induced in early DN stages, with TCF-1 having abundant expression thereafter and LEF-1 expression exhibiting further induction in post-selection DP thymocytes. Thus, TCF-1 and LEF-1 may act as a constant ‘inner drive’ toward the CD4+ T cell lineage.

TCF-1 contributes to specification to the CD4+ T cell lineage by direct positive regulation of Thpok. Thpok expression was diminished but not completely abrogated by TCF-1 deficiency. Hypomorphic Th-PK expression is known to cause redirection of CD4+ T cells to the CD8+ T cell lineage11, which indicates that a threshold of Th-PK expression is required for complete commitment to the CD4+ lineage. The diminished expression of Thpok in TCF-1-deficient post-selection DP and CD4+CD8β+ thymocytes was sufficient to reduce the output of CD4+ T cells and cause redirection to the CD8+ lineage on a B2m−/− background or with an MHC class II–restricted TCR. Although significant derepression of CD4 expression in Tcf7−/−/Lef1−/− CD8+ T cells precluded decisive gene-expression analysis of post-selection DP cells lacking both TCF-1 and LEF-1, we did observe more redirection from the CD4+ lineage to the CD8+ lineage in all models tested, which suggested that loss of TCF-1 and LEF-1 may have caused a greater reduction in Thpok expression.

TCF-1 deficiency also caused increased expression of Runx3d3d in the bipotent post-selection DP and CD4+CD8β+ thymocytes. However, this increase was most probably secondary to decreased Thpok expression, because the transgene encoding Th-PK suppressed Runx3d expression in both wild-type cells and Tcf7−/− cells. In addition, whereas ectopic expression of Th-PK restored the CD4+ T cell output of Tcf7−/− mice, deletion of Runx3 failed to do so. On the other hand, in mature TCRβhi thymocytes from Tcf7−/−/Lef1−/− mice, the transgene...
encoding Th-Pok was able to suppress the differentiation of CD8+ T cells and lineage redirection of CD4+ T cells to the CD8+ lineage in B2m-/- chimeric hosts but was inefficient in restoring CD4+ T cell numbers. This resembled the impaired generation of CD4+ SP thymocytes caused by CD4-Cre–mediated deletion of Gata3, which cannot be rectified by a transgene encoding Th-Pok. Therefore, loss of both TCF-1 and LEF-1 may have perturbed the expression of additional critical factors (other than Th-Pok) that may contribute to the promotion of CD4+ T cell differentiation.

Despite the intrinsic bias toward CD4+ lineage specification, Th-Pok expression and/or activity is opposed by Runx3 to ensure that DP thymocytes selected by MHC class I signaling commit to the CD8+ T cell lineage. It is well accepted that potent and persistent TCR signaling promotes the CD8+ lineage fate. Published studies have further demonstrated that intrathymic cytokine signaling, rather than TCR signaling, promotes the CD8+ lineage fate41,42. Indeed, IL-7 has been shown to induce the expression of Runx3, which in turn leads to silencing of Thpok43. Notably, IL-7-derived signals inhibit the expression of TCF-1 and LEF-1 (ref. 43). Given our new findings that TCF-1 and LEF-1 positively regulated Th-Pok, IL-7 signaling may prevent Th-Pok expression via various mechanisms, including repression of its positive regulators (such as TCF-1 and LEF-1) in addition to induction of its negative regulator, Runx3d.

Once a lineage decision is made, lineage-inappropriate genes must be silenced to ensure that the cell identity is inherited maintained. Runx factors have an important role in the silencing of Cd4 in cells committed to the CD8+ lineage44,45. Our results have revealed a critical contribution of TCF-1 and LEF-1 to the silencing of Cd4. TCF-1 and Runx3 physically interacted with each other and exhibited considerable synergy in silencing Cd4 in CD8+ T cells. Although it remains to be elucidated if TCF-1 is recruited to the Cd4 silencer directly by DNA element(s) or indirectly by Runx factors, Runx and TCF-LEF factors are both essential components of a protein complex that occupies the Cd4 silencer. Compelling evidence indicates that epigenetic mechanisms are involved in the inheritable silencing of Cd4 in CD8+ T cells45. TCF-LEF factors may contribute to the recruitment of histone-modification enzymes to the Cd4-silencing complex. Beyond its role in silencing Cd4, the cooperation between TCF-LEF and Runx factors might be essential for positive regulatory functions in other gene-regulatory contexts or might extend to different cell types, such as hematopoietic stem cells, as has been suggested46.

While TCF-1 and LEF-1 were required for the silencing of Cd4 in CD8+ T cells, early deletion of these factors does not cause aberrant expression of Cd4 in DN thymocytes18. In contrast, loss of the transcription factor Ikaros results in the opposite effect, causing derepression of Cd4 in DN thymocytes but not in CD8+ T cells47. Among the Runx factors, Runx1 has a more dominant role in the repression of Cd4 in DN cells, but Runx3 is more potent for the silencing of Cd4 in CD8+ T cells. Thus, the protein complex at the Cd4 silencer seems to undergo dynamic changes in components as thymocytes progress through various developmental stages. TCF-1 and LEF-1 are both expressed in multiple isoforms, with the full-length isoform being able to interact with β-catenin. The short isoforms have suppressive and/or dominant-negative functions16,21, and in fact the p33 isoform of TCF-1 interacts with Runx3. Future studies should determine if there is a ‘division of labor’ among the full-length and short isoforms of TCF-1 and LEF-1 in the silencing of Cd4 in CD8+ T cells and, by extension, in promoting Thpok expression in lineage choice.

Although ablation of the Runx complex causes derepression of both Cd4 and Thpok in CD8+ T cells10, loss of TCF-1 and LEF-1 specifically derepressed Cd4 but not Thpok. Consistent with that observation, ChIP-Seq analysis of TCF-1 in CD8+ T cells revealed no direct association of TCF-1 with the Thpok locus, and the previously defined Thpok silencer (the distal regulatory element) does not contain a TCF-LEF-binding consensus motif. Thus, the cooperation between TCF-LEF and Runx is highly dependent on gene context. In addition, the regulation of Thpok by TCF-1 at the GTE was restricted to the bipotent precursor cells, which indicates that the GTE may not be accessible to TCF-1 in T cells committed to the CD8+ lineage.

In summary, our studies have demonstrated a role switch for TCF-1 and LEF-1 in late stages of T cell development. These factors promoted the CD4+ T cell fate in DP and CD4+CD8+ thymocytes by positively regulating Th-Pok without directly involving Runx3. In T cells committed to the CD8+ lineage, however, TCF-1 and LEF-1 acted together with Runx3 to repress the lineage-inappropriate gene Cd4. Our findings reveal that the same transcriptional regulator contributes to fate decisions and the establishment of cell identity through distinct genetic and molecular ‘wiring’.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. GEO: TCF-1 ChIP-Seq data, GSE52070 (including GSM1258235 and GSM1258236).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

E.C.S. and S.Y. did experiments and analyzed the data; X.Z. and B.Z. did the coimmunoprecipitation experiments; B.H. and W.Y. analyzed the ChIP-Seq data under the supervision of K.T. and J.Z.; H.K. provided anti-TCF-1; H.-H.X. designed and supervised the study and, with F.C.S. and S.Y., wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animals. Mice in which Tcf7 was targeted were from Institut Clinique de la Souris (part of the International Knockout Mouse Consortium). Following rederivation at the animal-use facility of the University of Iowa, the mice were crossed with Rosa26-Flipase knock-in mice (Jackson Laboratory) for deletion of the LacZ-neo cassette flanked by the Frt sites (Supplementary Fig. 1); this converted the targeted allele into a loxp-flanked allele (Tcf7fl/fl). The Tcf7+/− mice have been described18, Runx3+/− mice were from the Jackson Laboratory; B2m−/− and H2-Ab1−/− mice were from Taconic; and mice expressing the transgene encoding Th-Pok were provided by R. Bosselut29. All mice were analyzed at 5–10 weeks of age, and both sexes were included without randomization or ‘blinding’. All chimeras were analyzed within 6–10 weeks of BM transplantation. Throughout all the experiments in this study, we found that mice heterozygous for the Tcf7−/−, Lef1−/−, or Runx3-targeted allele alone or in various combinations were not detectably different from wild-type mice in terms of thymic architecture and thymocyte developmental patterns, as determined by flow cytometry (data not shown). For analysis of Tcf7+/−CD4-Cre (Tcf7−/+− mice), their Tcf7+/−CD4-Cre and Tcf7+/−CD4-Cre littermates served as controls. For analysis of Tcf7fl/flCD4-Cre (Tcf7−/+− mice), their Tcf7+/−Lef1+/−− CD4-Cre, Tcf7+/−Lef1−/−− CD4-Cre, Tcf7fl/flLef1+/−− CD4-Cre and Tcf7fl/flLef1−/−− CD4-Cre littermates served as controls. The same principle applied to experiments that incorporated the use of the H2-Ab1−/− background, transplantation into B2m−/− hosts or the transgene encoding Th-Pok. For analysis of Tcf7fl/flRunx3−/−CD4-Cre (Tcf7−/−Runx3−/− mice), their Tcf7+/−Runx3−/− Cd4-Cre, Tcf7+/−Runx3−/− Cd4-Cre, Tcf7+/−Runx3−/− Cd4-Cre and Tcf7fl/flRunx3−/− Cd4-Cre littermates served as controls. For analysis of Tcf7fl/flLef1+/−−Runx3−/− Cd4-Cre (Tcf7−/−Lef1+/−− Runx3−/− mice), their littermates with genotypes ranging from Tcf7+/−Lef1−/−−Runx3−/− Cd4-Cre to Tcf7fl/flLef1+/−−Runx3−/− Cd4-Cre served as controls. All mouse experiments were done with protocols approved by the Institutional Animal Use and Care Committee of the University of Iowa.

Flow cytometry. Single-cell suspensions were prepared from thymus and spleen and cell surfaces were stained as described18. The following fluorochrome-conjugated antibodies were from eBioscience: anti-CD4 (RM4-5), anti-CD8 (H30-1.3), anti-TCRβ (H57-597), anti-CD24 (M1/69), anti-CD99 (H1.2F3), anti-CD45.2 (104), anti-B20.1 (RA3-6B2), anti-Gr-1 (53-6.7), anti-TCRβ (B20.1), anti-interferon-γ (XMG1.2), anti-IL-2 (JES6-5H4) and streptavidin (48-4317-82; eBioscience). Anti-CD8α (H35-17.2), anti-CD24 (M1/69), anti-Gr-1 (53-6.7) was from BD Biosciences. Anti-galectin-3 (GB-111) and mouse IgG1 (MG104) were from Life Technologies. Data were collected on a FACSVerse (BD Biosciences) and were analyzed with FlowJo software (version X; TreeStar).

Generation of chimeras. B2m−/− mice were crossed with the B6.6J strain to acquire homozygous expression of CD45.1 and the progeny were used as recipients. Whole BM cells were isolated from various donors, and 2 × 10^6 cells were transplanted into irradiated recipients by injection into the tail vein.

 Luciferase assay. The 473-bp wild-type GTE was amplified by PCR, and the mutated GTE was synthesized (GenScript) with the proper flanking restriction enzyme sites. Those segments were cloned into the pGL3 promoter vector (Promega) via the Kpn1 and Nhel sites upstream of the SV40 promoter or via the BamHI and Sall sites downstream of the gene encoding luciferase. The reporter constructs were transfected into EL4 cells by electroporation (GenePulser Xcell; Bio-Rad) or into 293T cells through the use of Lipofectamine 2000 (Life Technologies) by standard protocols48. The plasmid pRL-TK, which expresses renilla luciferase driven by the promoter of a gene encoding thymidine kinase, was cotransfected as an internal control. Then, 48 h later, cell lysates were extracted and the luciferase activity was analyzed with the Dual-Luciferase Reporter Assay System (Promega).

Gene-expression assay. Target cell populations were sorted from thymocytes or splenocytes and RNA extraction, reverse-transcription and quantitative PCR were done as described18 (primer sequences, Supplementary Table 1).

ChIP. Post-selection DP and CD4+CD8− thymocytes, mature CD8+ or CD4+SP thymocytes, or splenic CD8+ T cells were sorted from wild-type C57BL/6 or Tcf7−/− mice. The cells were crosslinked for 5 min with 1% formaldehyde in medium, were processed with a trueChIP Chromatin Shearing Reagent Kit (Covaris) and were sonicated for 5 min on a Covaris S2 ultrASONICATOR. The sheared chromatin was immunoprecipitated with anti-TCF-1 (prepared in-house)39 or IgG (2729; Cell Signaling Technology) and were washed as described18. The immunoprecipitated DNA segments were used for library construction or quantification by PCR. For calculation of enrichment for TCF-1 binding in a given cell type by ChIP followed by PCR (primers, Supplementary Table 1), the result for each ChIP analysis with anti-TCF-1 was first normalized to that of the corresponding ChIP analysis with IgG, and the signal at a target region was then normalized to that at the Hprt or Gapdh promoter region.

ChIP-Seq and data analysis. DNA segments from ChIP were end-repaired and ligated to indexed Illumina adaptors, followed by amplification by PCR with a low number of cycles (18–21 cycles). The resulting libraries were sequenced with the Illumina Hiseq-2000 platform. Sequencing ‘reads’ were mapped to the mm9 National Center for Biotechnology Information assembly of the mouse genome with Bowtie software for the alignment of short DNA sequences (version 0.12.5). The mapping statistics for ChIP were as follows: for ChIP-Seq with anti-TCF-1, total reads, 24,390,268, and mapped reads, 23,262,097 (95.4%); for ChIP-Seq with IgG, total reads, 48,752,154, and mapped reads, 46,601,382 (95.6%).

The MACS algorithm34 was used for peak calling with two sets of cutoffs. The first set used the criteria of enrichment of four-fold or more, a P value of <10−5 and false-discovery rate of <5% and thus identified stringent TCF-1-binding peaks. The second set used the same criteria for enrichment and false-discovery rate but a P value of <10−3, which identified additional permissive TCF-1-binding peaks. The TCF-1 peaks identified were analyzed with the MEME-ChIP suite of motif-based sequence-analysis tools (version 4.9.0) to define consensus DNA-binding motifs49. Peak sequences were ‘padded’ with 200-bp genomic sequences on each side for motif scanning. The Patser program to find locations of patterns in sequence (version 3b) was used to scan each TCF-1 peak region for the co-occurrence of TCF-1- and Runx-binding sites50.

Immunoprecipitation and immunoblot analysis. The Tcf7 and Runx33d coding sequence was amplified from CD8+ T cell cDNA and was cloned into the MigR1 retrovector vector, and sequence encoding a 3× Flag tag was placed in-frame on sequence encoding the amino terminus of TCF-1. The expression plasmids were transfected into 293T cells with Lipofectamine 2000 (Life Technologies) and, 48 h later, cell lysates were extracted and then incubated overnight with 2 μg of anti-Runx3 (S27327; R&D Systems), anti-Flag (M2; Sigma-Aldrich) or mouse IgG (sc-2025; Santa Cruz Biotechnology), followed by 2 h of incubation with Dynabeads Protein G (Life Technologies). After proper washing, the immunoprecipitated samples were analyzed by immunoblot with anti-Myc (7D10; Cell Signaling Technologies), anti-Flag (identified above) or anti-Runx3 (identified above).

For analysis of the efficiency with which TCF-1 and LEF-1 were deleted, pre- and post-selection DP thymocytes (5 × 10^5 each) were sorted, then their lysates were probed with anti-TCF-1 (C46C7; Cell Signaling Technology), anti-LEF-1 (C18A7; Cell Signaling Technology) or anti-β-actin (loading control; I-19; Santa Cruz Biotechnology).

Statistical analysis. Data sets were analyzed with the Student's t-test with a two-tailed distribution assuming equal sample variance.

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