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Transcriptional reprogramming of mature CD4+ helper T cells generates distinct MHC class II–restricted cytotoxic T lymphocytes

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TCRαβ thymocytes differentiate into either CD8αβ+ cytotoxic T lymphocytes or CD4+ helper T cells. This functional dichotomy is controlled by key transcription factors, including the helper T cell master regulator ThPOK, which suppresses the cytolytic program in major histocompatibility complex (MHC) class II–restricted CD4+ thymocytes. ThPOK continues to repress genes of the CD8 lineage in mature CD4+ T cells, even as they differentiate into effector helper T cell subsets. Here we found that the helper T cell fate was not fixed and that mature, antigen-stimulated CD4+ T cells terminated expression of the gene encoding ThPOK and reactivated genes of the CD8 lineage. This unexpected plasticity resulted in the post-thymic termination of the helper T cell program and the functional differentiation of distinct MHC class II–restricted CD4+ cytotoxic T lymphocytes.

CD4+ T cells are commonly classified as 'helper' T cells on the basis of their roles in providing help to promote or dampen cellular and humoral immune responses. In contrast, CD8αβ+ cytotoxic T lymphocytes (CTLs) provide direct protective immunity by killing infected or transformed cells. The helper T cell program is initially induced during thymic development, during which thymocytes expressing a major histocompatibility complex (MHC) class II–reactive T cell antigen receptor (TCR) develop into the CD4+ helper T cell lineage, whereas thymocytes with specificity for MHC class I differentiate into the CD8+ CTL lineage. The functional programming, which coincides with but does not depend on the MHC restriction or expression of the coreceptor CD4 or CD8αβ, is controlled by the action and counteraction of key transcription factors. Together with Tox and GATA-3, the helper T cell transcription factor ThPOK (cKrox; encoded by *Zbtb7b* (called '*Thpok*' here)) first induces the CD4+ helper T cell fate and prevents thymocytes from differentiating into CD8+ CTLs[1–6]. Runx3, a member of the Runx family of transcription factors, has the opposite effect and terminates CD4 expression while promoting differentiation into the CTL lineage[7,8]. The CD8-CD4 lineage dichotomy persists in the periphery for mature T cells, in which ThPOK continues to suppress the cytotoxic fate of MHC class II–restricted CD4+ T cells even as they differentiate into effector helper T cell subsets controlled by additional transcription factors[6].

That lineage separation, however, is not all encompassing, and reports have repeatedly indicated the presence of CD4+ T cells with cytolytic functions in various species, including humans and rodents[9–12]. At steady state, populations of the effector cells that reside in the intestine as intraepithelial lymphocytes (IELs) show enrichment for cytotoxic T cells, including CD4+ T cells[13–15], whereas under inflammatory conditions, including viral infections, autoimmune disorders and in response to tumor antigens, many cytolytic CD4+ T cell populations expand in the blood and peripheral tissues[9–12,16,17]. Their widespread abundance and participation in various beneficial as well as pathogenic adaptive immune responses[9–12,16,17] underscore the physiological relevance of cytolytic CD4+ effector cells. However, the generation of convincing evidence directly linking them to specific

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aspects of the adaptive immune response has been difficult, as they have been viewed merely as functional variants of the well-defined T helper type 1 (T_H1) subtype[12,18]. The lack of a defined gene signature has also greatly impaired progress in elucidating the biology of cytolytic CD4$^+$ T cells and in designing clinical strategies that specifically target these cells in various diseases.

Here we provide proof indicating that cytotoxic CD4$^+$ T cells represent a distinct subset of effector cells that can be defined by the absence of the master regulator ThPOK, which maintains the helper T cell fate in all subsets of classical CD4$^+$ helper T cells by continuously suppressing the CD8$^+$ CTL lineage program[6]. Nevertheless, we also found that the ThPOK$^-$ CD4$^+$ CTLs originated from ThPOK-expressing progenitor cells that initially committed to the ThPOK-controlled helper T cell lineage during thymic selection. Our findings therefore challenge the view that the helper T cell program in CD4$^+$ T cells is fixed and show that mature CD4$^+$ T cells can lose ThPOK expression post-thymically and can be functionally reprogrammed to become MHC class II–restricted CTLs. We also identify the *Thpok* silencer as the transcriptional switch that terminated *Thpok* transcription and by default drove the derepression of the CTL program in mature CD4$^+$ effector cells. At steady state, CD4$^+$ CTLs remained immunologically quiescent even in the continuous presence of their cognate antigens. However, in response to restimulation in the context of interleukin 15 (IL-15), CD4$^+$ CTLs greatly increased their inflammatory and cytolytic functions and differentiated into potent killer effector cells. Overall, our data demonstrate that CD4$^+$ CTLs are not a simple variant of classical ThPOK-controlled T_H1 cells but instead are distinct functional MHC class II–restricted effector cells that can be characterized by the loss of ThPOK expression and the derepression of aspects of the gene-expression program of the CD8$^+$ CTL lineage.

RESULTS

Not all mature CD4$^+$ T cells express ThPOK

The reported cytolytic activity of mature CD4$^+$ T cells is inconsistent with the idea that ThPOK continuously suppresses the CTL program in all mature, MHC class II–restricted CD4$^+$ T cells[6] and suggests that these cells might not be under the negative control of ThPOK. To investigate this, we analyzed ThPOK expression in mature T cells isolated from reporter mice with sequence encoding green fluorescent protein (GFP) knocked in to the *ThPOK* locus (*Thpok*-GFP mice)[19]. As expected, CD4$^+$ *Thpok*-GFP lymphocytes isolated from the spleen or mesenteric lymph nodes (mLNs), which are mostly naive T cells, were GFP$^+$ (**Fig. 1a**), which indicated that they all expressed ThPOK, as is typical of mature cells of the CD4$^+$ helper T cell lineage. Conversely, all cells in the CD8$^+$ fraction were GFP$^-$ (**Fig. 1a**), consistent with the absence of ThPOK expression in cells of the CTL lineage. Unexpectedly, many of the *Thpok*-GFP CD4$^+$ effector T cells that accumulated in the intestine at steady state were GFP$^-$ (**Fig. 1b**,**c**), which indicated that like their CD8$^+$ counterparts, they did not express ThPOK. Notably, most of the GFP$^-$ CD4$^+$ cells were in the subset of double-positive (DP) IELs that coexpressed CD4 and CD8α without CD8β[20] (**Fig. 1b–d**). Consistent with the lack of ThPOK-mediated suppression, these CD4$^+$CD8α$^+$CD8β$^-$ cells also had functional features similar to those of mature CD8$^+$ CTLs, including abundant expression of granzyme (**Fig. 1e**,**f**) and substantial expression of the activation-induced degranulation marker CD107a (LAMP-1), a glycoprotein present in the membrane of cytotoxic granules and exposed on the surface of activated cytolytic cells[21] (**Fig. 1g**,**h**). The induction of CD107a by the DP subset was similar to that of typical TCRαβ$^+$ CD8$^+$ CTLs, whereas activated CD4$^+$CD8α$^-$ single-positive (SP) IELs or helper T cells from the spleen did not induce this cytolytic marker

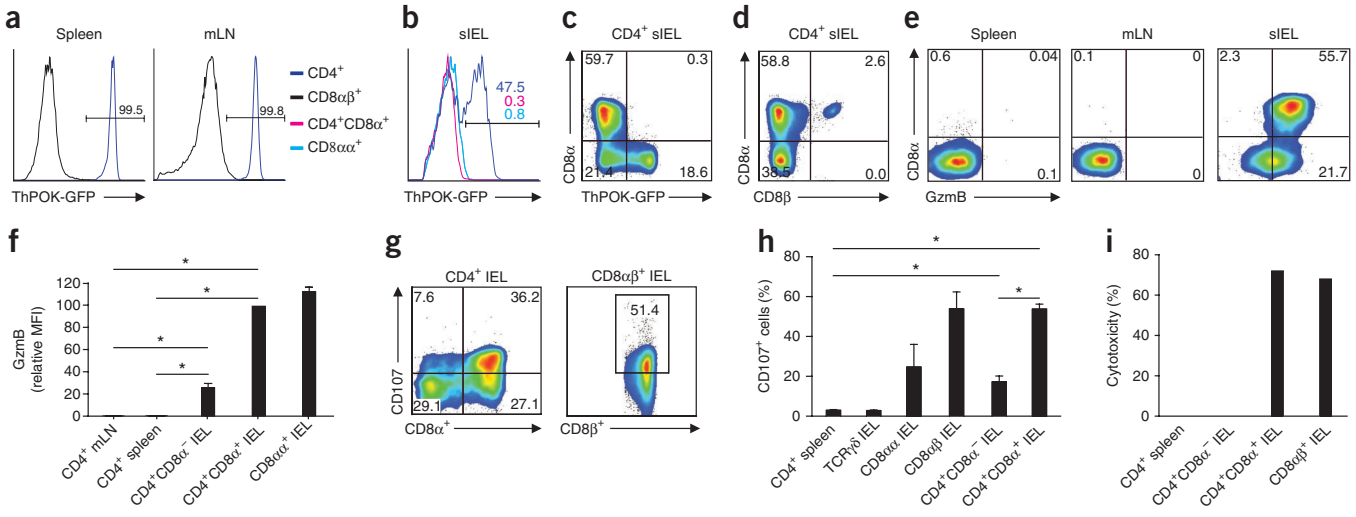

Figure 1 Some mature CD4$^+$ T cells do not maintain ThPOK expression in the periphery. (**a**) Frequency of GFP$^+$ cells (numbers above bracketed lines) among gated CD45$^+$TCRβ$^+$ lymphocytes isolated from the spleen and mLNs of naive *Thpok*-GFP reporter mice ($n = 4$ or 5). (**b**) Frequency of GFP$^+$ cells (numbers above bracketed lines) among gated CD45$^+$TCRβ$^+$ IELs isolated from the small intestine (sIEL) of naive *Thpok*-GFP reporter mice ($n = 4$ or 5). (**c**) Cell surface staining for CD8α and GFP on gated CD45$^+$TCRβ$^+$CD8β$^-$CD4$^+$ IELs as in **b**. Numbers in quadrants indicate percent cells in each throughout. (**d**) Cell surface staining for CD8α and CD8β on gated CD45$^+$TCRβ$^+$CD4$^+$ IELs as in **b**. (**e**) Cell surface staining for CD8α and intracellular for staining granzyme B (Gzmb) on or in gated CD45$^+$TCRβ$^+$CD8β$^-$CD4$^+$ lymphocytes isolated from the spleen and mLNs and IELs from the small intestine of naive wild-type C57BL/6 mice ($n = 4$ or 5). (**f**) Mean fluorescence intensity (MFI) of granzyme B in CD4$^+$, CD4$^+$CD8α$^+$ or CD8αα$^+$ T cells among gated CD45$^+$TCRβ$^+$CD8β$^-$ lymphocytes from the spleen and mLNs and IELs of naive wild type C57BL/6 mice ($n = 4$ or 5); results are presented relative to those of the CD4$^+$CD8α$^+$ subset, set as 100%. $^*P < 0.05$ (analysis of variance (ANOVA) with Bonferroni's post-test). (**g**) Frequency of CD107$^+$ cells (number in outlined area at right) among total gated CD45$^+$TCRβ$^+$CD8β$^-$CD4$^+$ and CD45$^+$TCRβ$^+$CD8αβ$^+$ IELs after stimulation with antibody to TCRβ. (**h**) Frequency of CD107$^+$ cells among CD4$^+$ splenocytes or TCRγδ$^+$, CD8αα$^+$, CD8αβ$^+$, CD4$^+$CD8α$^-$ and CD4$^+$CD8α$^+$ IEL subsets after stimulation with antibody to TCRβ ($n = 4$ replicates). $^*P = 0.02$ (nonparametric two-tailed Mann-Whitney test). (**i**) Cytotoxicity of lymphocyte subsets, assessed by lactate dehydrogenase–release assay. Data are representative of two (**a**,**b**), three (**c**–**f**) or four (**g**–**i**) independent experiments (error bars (**f**,**h**,**i**), s.e.m.).

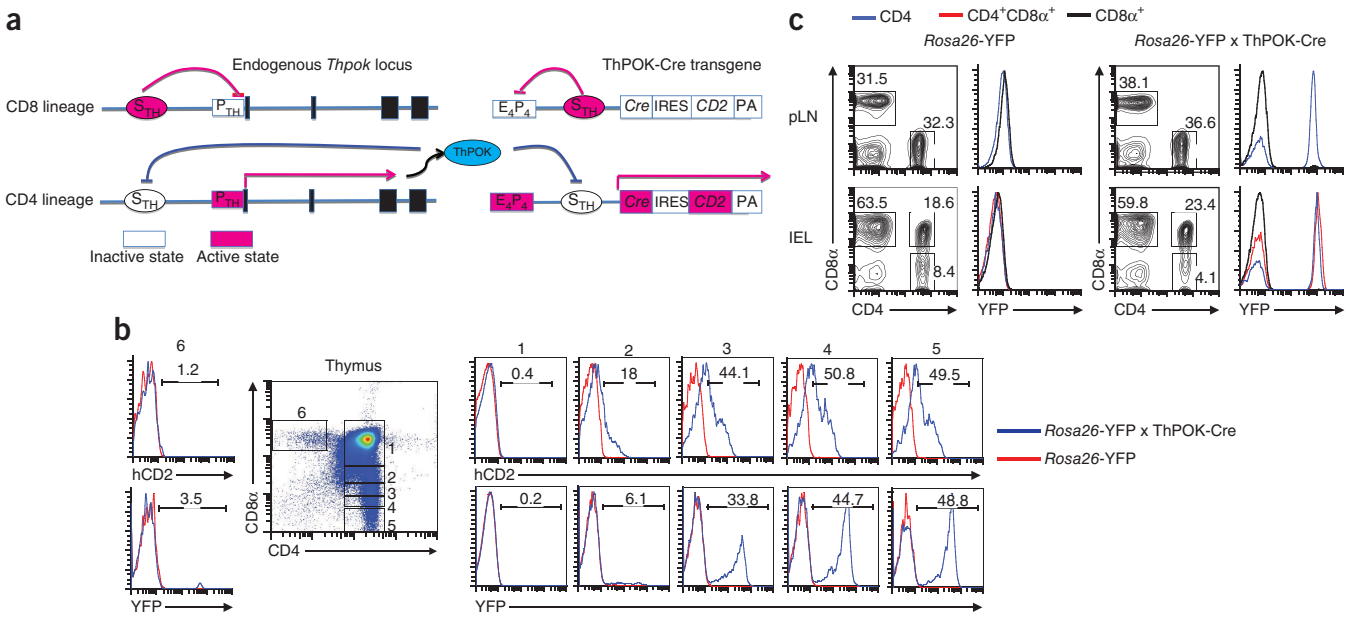

Figure 2 Mature ThPOK⁻ CD4⁺ T cells are the progeny of ThPOK-expressing thymocytes. (**a**) Endogenous *Thpok* (left) and the transgene encoding ThPOK-Cre (right) in thymocytes of the CD8 and CD4 lineages. S_{TH}, *Thpok* silencer; P_{TH}, *Thpok* promoter; E_4P_4, *Cd4* enhancer-promoter; IRES, internal ribosomal entry site; *CD2*, human gene encoding CD2; PA, poly(A) tail. (**b**) Staining for CD8α and CD4 (middle) and for human CD2 (hCD2) and YFP (left and right) in thymocytes gated on expression of CD4 and CD8α (gates 1–6 (middle), where 1 represents immature DP thymocytes and 5 and 6 represent mature CD4⁺ and CD8⁺ SP thymocytes, respectively), from *Rosa26*-YFP mice and *Rosa26*-YFP mice expressing ThPOK-Cre (with the transgene in **a**; *Rosa26*-YFP × ThPOK-Cre). Numbers above bracketed lines (left and right) indicate percent cells positive for human CD2 (ThPOK-Cre⁺; top rows) or YFP⁺ cells (bottom rows). (**c**) Staining for CD4 and CD8α (contour plots) and YFP (histograms) in cells from the peripheral lymph nodes (pLN) and IELs from the small intestine of mice as in **b**, gated on TCRβ⁺CD4⁺ lymphocytes. Numbers adjacent to outlined areas indicate percent cells in each. Data are representative of at least three independent experiments.

(**Fig. 1g**,**h**). Moreover, activated DP cells also effectively killed target cells *in vitro*, as measured by the release of lactate dehydrogenase after lysis of target cells (**Fig. 1i** and **Supplementary Fig. 1a**). In sum, these data demonstrated that in normal mice, not all CD4⁺ effector cells expressed ThPOK and, furthermore, that those ThPOK⁻ CD4⁺ lymphocytes expressed CD8α (but not CD8β) and had cytolytic activity that closely resembled that of mature CD8⁺ CTLs.

Mature ThPOK⁻ CD4⁺ T cells derive from ThPOK⁺ thymocytes

ThPOK is the master regulator of the helper T cell lineage and is first expressed in the thymus, where it counteracts Runx3 and suppresses the CTL fate of MHC class II–restricted thymocytes[4–6]. The absence of ThPOK expression associated with cytotoxicity in mature CD4⁺ T cells could suggest that they might have originated from ThPOK⁻ progenitors. To investigate this, we designed a fate-mapping mouse model in which we tracked previous ThPOK expression in mature T cell subsets (**Fig. 2a**). Inactivation of *Thpok* transcription in MHC class I–specific thymocytes of the CD8⁺ CTL lineage is mediated by repressive factors, such as Runx proteins, that bind to the *Thpok* silencer DNA element[22,23]. In MHC class II–restricted cells, however, ThPOK itself binds to the *Thpok* silencer and prevents Runx-mediated silencing, which results in a positive feedback loop with continuous expression of *Thpok* (**Fig. 2a**). A fate marker that reports any previous activity of the *Thpok* silencer in thymocytes is therefore an accurate reporter for the lineage origin and initial commitment of mature CD4⁺ T cells. On the basis of that rationale, we designed a mouse strain with transgenic expression of Cre recombinase, 'ThPOK-Cre', in which a *Thpok* silencer inserted into a *Cd4* enhancer-promoter locus controls expression of a transgene encoding Cre (**Fig. 2a**). In contrast to the widely used strain of mice that express Cre under the control of the *Cd4* enhancer-promoter starting at the CD4⁺CD8αβ⁺ thymocyte stage, in ThPOK-Cre mice, the *Thpok* silencer prevents *Cd4* enhancer-promoter activity in immature thymocytes and cells of the CD8 lineage, which results in Cre expression exclusively in mature CD4⁺ SP thymocytes committed to the helper T cell lineage (**Fig. 2a**). Consistent with that, in progeny of a cross of ThPOK-Cre mice with reporter mice expressing yellow fluorescent protein (YFP) from the ubiquitous *Rosa26* promoter (*Rosa26*-YFP), we found that only cells derived from thymocytes that were committed to the CD4⁺ helper T cell lineage were marked by the expression of the YFP reporter after Cre-mediated recombination (**Fig. 2b**,**c**). In those progeny of the *Rosa26*-YFP × ThPOK-Cre cross, CD8⁺ SP thymocytes and mature peripheral CD8⁺ T cells, including CD4⁻CD8⁺ mucosal T cells, did not express YFP, whereas, as expected, most CD4⁺ SP lymph node T cells and IELs were YFP⁺. Notably, CD8α-expressing CD4⁺ IELs were also YFP⁺, to an extent similar to that of conventional CD4⁺ SP cells (**Fig. 2b**,**c**), which indicated that they had induced ThPOK-dependent Cre expression and therefore that the mature ThPOK⁻ CD4⁺ T cells must have been derived from progenitor cells that expressed ThPOK at an earlier stage.

ThPOK⁺CD4⁺ helper T cells lose ThPOK expression

The idea that the mature ThPOK⁻ CD4⁺ cells previously expressed ThPOK, together with the observation that those cells mainly accumulated among effector cells, suggested that the loss of ThPOK expression might have been the result of a post-thymic activation or maturation process that mature T cells undergo in the periphery. To assess this, we adoptively transferred highly purified

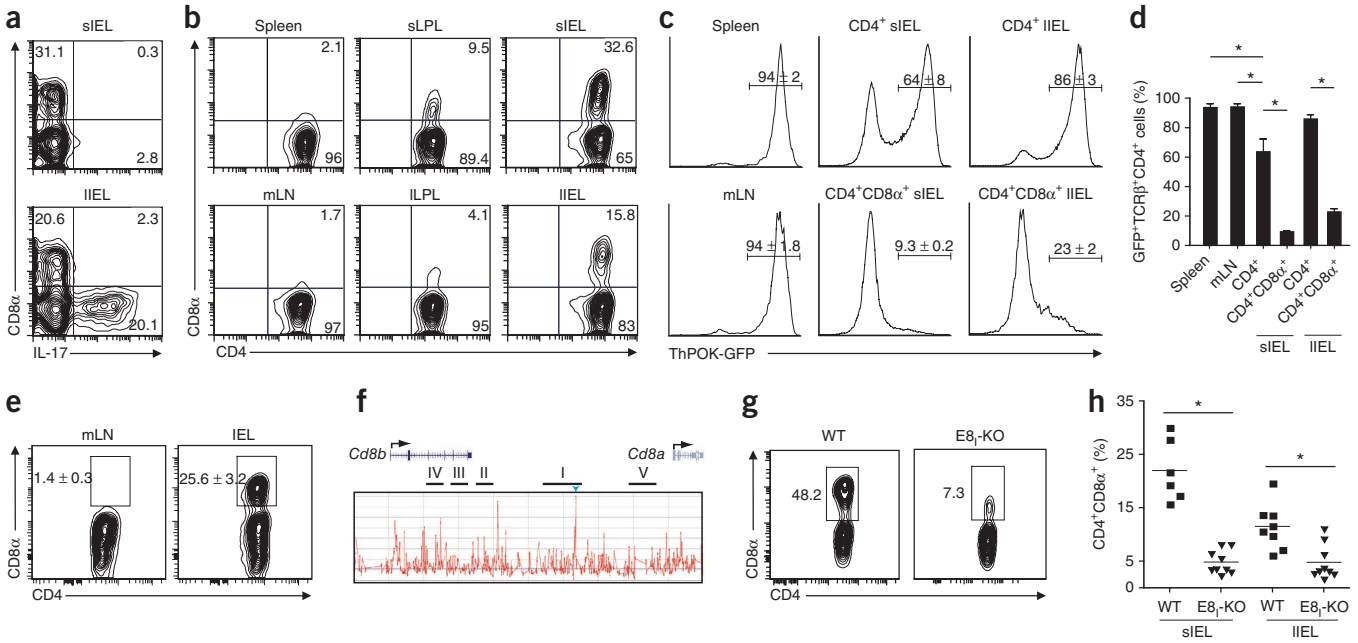

Figure 3 ThPOK⁻ CD4⁺ effector cells lost ThPOK as mature cells in the periphery. (**a**) Staining of CD8α and intracellular IL-17 in gated CD45⁺TCRβ⁺CD4⁺ IELs from the small intestine (sIEL) and large intestine (IIEL) of *Rag1*$^{-/-}$ recipient mice 8 weeks after adoptive transfer of naive CD45RB^{hi}TCRβ⁺CD8α⁻CD25⁻CD4⁺ spleen T cells. (**b**) Staining of CD8α and CD4 in gated CD45⁺TCRβ⁺CD4⁺ T cells from the spleen and mLNs, lymphocytes from the lamina propria of the small intestine (sLPL) and large intestine (lLPL) and IELs from the small intestine and lage intestine of *Rag1*$^{-/-}$ recipient mice 8 weeks after adoptive transfer of naive CD45RB^{hi}TCRβ⁺CD8α⁻CD25⁻CD4 spleen T cells. (**c**) Expression of *Thpok*-GFP in gated CD45⁺TCRβ⁺ CD4⁺ (SP) or CD4⁺CD8α⁺CD8β⁻ (DP) lymphocytes isolated from various tissues (as in **a**,**b**) of *Rag1*$^{-/-}$ recipient mice 8 weeks after transfer of sorted CD45RB^{hi}TCRβ⁺CD8α⁻CD25⁻CD4⁺ spleen T cells from *Thpok*-GFP donor mice. Numbers above bracketed lines indicate percent GFP⁺ cells (mean ± s.e.m.). (**d**) Frequency of GFP⁺ cells among gated CD45⁺TCRβ⁺ CD4⁺ (SP) or CD4⁺CD8α⁺CD8β⁻ (DP) lymphocytes from spleen or mLNs or IELs of *Rag1*$^{-/-}$ recipients after transfer of sorted naive CD45RB^{hi}TCRβ⁺CD8α⁻GFP⁺CD25⁻CD4⁺ spleen T cells from *Thpok*-GFP donor mice. *$P < 0.001$ (ANOVA and Bonferroni's post-test). (**e**) Surface staining for CD8α on retransferred sorted (>99.7% purity) CD45⁺TCRβ⁺CD8α⁻CD4⁺ donor IELs isolated from mLNs (pooled from three recipient mice) and IELs of the small intestine another *Rag1*$^{-/-}$ recipient 8 weeks after the second transfer. Numbers adjacent to outlined areas indicate percent CD8α⁺ cells (mean ± s.e.m.). (**f**) ChIP with tiling array of CD4⁺ SP thymocytes from FH-ThPOK mice. I–V, *Cd8* enhancers; green arrowhead indicates binding of ThPOK to the E8$_I$ region. (**g**) Frequency of CD8α⁺ cells (numbers adjacent to outlined areas) among CD45⁺TCRβ⁺CD8β⁻CD4⁺ IELs isolated from a wild-type mouse (WT) and an E8$_I$-deficient mouse (E8$_I$-KO). (**h**) Frequency of CD8α⁺CD4⁺ IELs from *Rag1*$^{-/-}$ recipient mice 8 weeks after transfer of wild type or E8$_I$-deficient CD45RB^{hi}TCRβ⁺CD8α⁻CD25⁻CD4⁺ spleen T cells. Each symbol represents an individual mouse; small horizontal lines indicate the mean. *$P < 0.001$ (ANOVA and Bonferroni's post-test). Data are representative of three independent experiments (**a**–**d**; error bars (**d**), s.e.m.), two independent experiments (**e**,**f**,**h**) or three independent experiments with four or five mice per genotype (**g**).

ThPOK-expressing GFP⁺ naive *Thpok*-GFP CD4⁺ T cells into recipient mice deficient in recombination-activating gene 1 (*Rag1*$^{-/-}$ mice). The lymphopenic conditions in this model induced strong proliferation and differentiation of the donor cells, which accumulated mainly as cells of the T_H17 subset of helper T cells (characterized by the expression of the transcription factor RORγt and the cytokine IL-17) in the large intestine, and notably, also as DP effector cells, especially in the small intestine of the *Rag1*$^{-/-}$ hosts (**Fig. 3a**,**b**). Donor cells in the spleen and mLNs were GFP⁺ (**Fig. 3c**), which indicated that they continued to express ThPOK. In contrast, many of the *Thpok*-GFP CD4⁺ T cells that accumulated as effector cells in the intestine did not have detectable expression of GFP (**Fig. 3c**), which indicated that they had substantially diminished or complete loss of ThPOK expression. Consistent with the observations obtained with immunologically replete mice, the loss of GFP expression was again greatest in those CD4⁺ cells that also reinduced CD8α expression, although a small fraction of CD4⁺ SP IELs were also GFP⁻ (**Fig. 3c**,**d**). Serial transfer of those SP donor cells into a second set of *Rag1*$^{-/-}$ recipients generated many more DP cells (**Fig. 3e**), which indicated that the reexpression of CD8α on mature CD4⁺ T cells followed the loss of ThPOK expression and further indicated that the *Cd8a* locus in conventional CD4⁺ helper T cells might be constitutively suppressed by ThPOK. To analyze this, we used chromatin immunoprecipitation (ChIP) combined with tiling arrays of cells from genetically engineered mice that express Flag-hemagglutinin epitope–tagged ThPOK (FH-ThPOK) from the *Thpok* locus[19]. FH-ThPOK associated with the E8$_I$ enhancer element[24] in the *Cd8a* locus in SP CD4⁺ thymocytes (**Fig. 3f**). We also confirmed that result in mature CD4⁺ SP T cells by ChIP assay (**Supplementary Fig. 2a**), which suggested that in mature CD4⁺ thymocytes and lymphocytes, ThPOK prevented CD8α expression by direct suppression of the E8$_I$ enhancer element in the *Cd8a* locus, and also that the expression of CD8α without CD8β in mature ThPOK⁻ CD4⁺ T cells was driven by derepression of the E8$_I$ enhancer. In support of that finding, both the frequency of CD8α-expressing CD4⁺ IELs and CD8 expression itself were much lower in E8$_I$-deficient mice than in wild-type mice (**Fig. 3g**), a finding also true for the progeny of E8$_I$-deficient cells versus wild type donor cells in *Rag1*$^{-/-}$ recipients of an equal number of both genotypes of donor cells (**Fig. 3h** and **Supplementary Fig. 2b**). Overall the data indicated that naive ThPOK⁺ CD4⁺ helper T cell cells might have lost expression of ThPOK in the periphery and consequently regained expression of ThPOK-suppressed genes such as *Cd8a*.

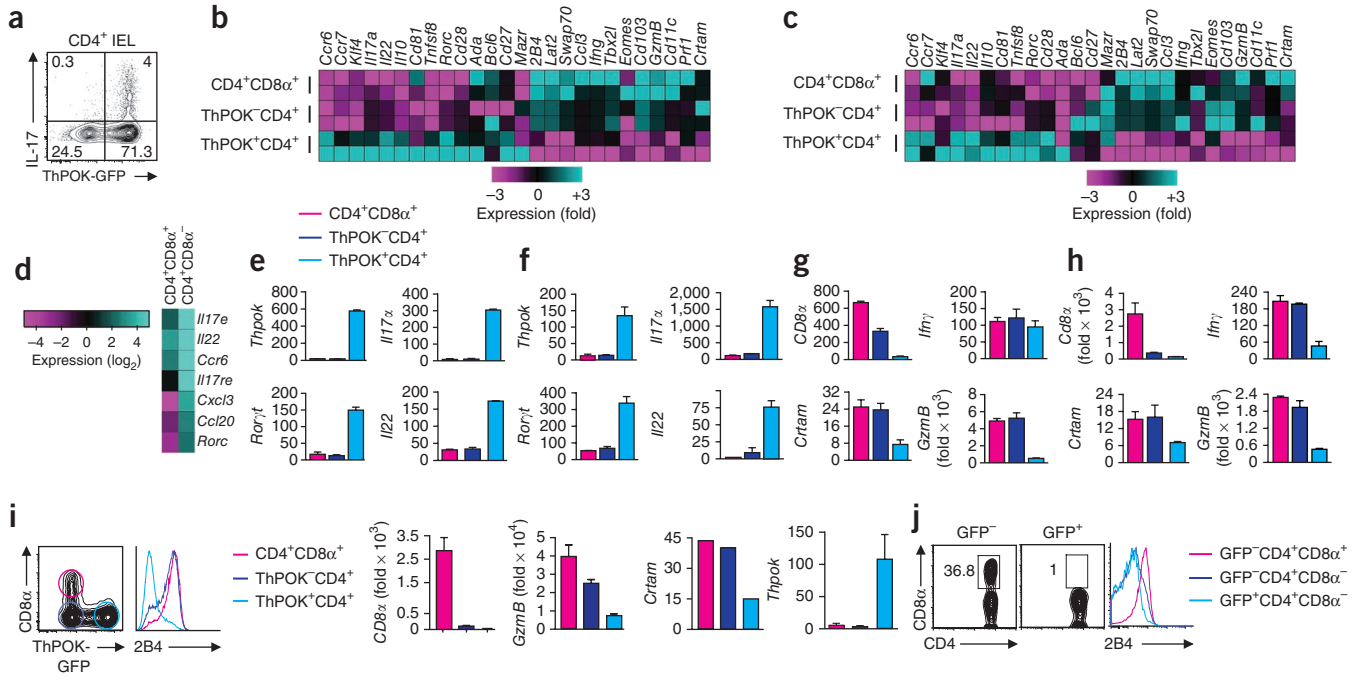

Figure 4 Activated CD4+ helper T cells that lose ThPOK expression differentiate into CTLs. (**a**) Intracellular staining for IL-17 and GFP in gated CD45+TCRβ+CD4+ IELs from *Rag1*$^{-/-}$ recipients of sorted naive CD45RB^{hi}TCRβ+CD8α−GFP+CD25−CD4+ spleen T cells from *Thpok*-GFP donor mice, assessed 8 weeks after cell transfer. (**b**) Gene-expression microarray analysis of mRNA from sorted CD45+TCRβ+ ThPOK+CD8α−CD4+, ThPOK−CD8α−CD4+ and ThPOK−CD8α+CD4+ IELs from naive *Thpok*-GFP reporter mice; results are relative to the change in expression in the ThPOK+CD4+CD8α− subset. (**c**) Gene-expression microarray analysis of mRNA from sorted CD45+TCRβ+ ThPOK+CD8α−CD4+, ThPOK−CD8α−CD4+ and ThPOK−CD8α+CD4+ IELs from *Rag1*$^{-/-}$ recipients of sorted naive TCRβ+CD8α−GFP+CD45RB^{hi}CD25−CD4+ spleen T cells from *Thpok*-GFP donor mice (presented as in **b**). (**d**) Heat map of normalized expression of T_H17 signature genes in donor CD4+ T cells as in **c**, determined by microarray analysis as in **b**. (**e**,**f**) Quantitative real-time PCR analysis of mRNA from T_H17-associated genes in sorted T cell subsets as in **c** (**e**) or **d** (**f**); results are presented relative to those for *Rpl32*. (**g**,**h**) Quantitative real-time PCR analysis of mRNA from CTL-associated genes in sorted T cell subsets as in **c** (**g**) or **d** (**h**), presented as in **e**,**f**. (**i**) Expression of ThPOK-GFP and 2B4 in gated CD45+TCRβ+CD4+ IELs as in **a** (left), and quantitative real-time PCR analysis of mRNA encoding CD8α, granzyme B, CRTAM and ThPOK in IELs sorted for (CD45+TCRβ+CD4+) GFP+, GFP−CD8α− and GFP−CD8α+ subsets isolated from *Rag1*$^{-/-}$ recipients as in **a** ($n = 4$; right). (**j**) Staining for CD4 and CD8α (left and middle) and 2B4 (right) in gated TCRβ+CD4+ IELs from *Rag1*$^{-/-}$ recipients ($n = 3$) of untransfected (GFP−) or ThPOK-transfected (GFP+) TCRβ+CD8α−CD45RB^{hi}CD25−CD4+ donor spleen T cells. Data are representative of three (**a**,**e**–**j**) or two (**b**–**d**) independent experiments (mean and s.e.m. in **e**–**i**).

ThPOK loss coincides with CTL differentiation

Although subsets of polarized CD4+ effector cells are controlled by unique transcription factors, such as T-bet for T_H1, GATA-3 for T_H2 and RORγt for T_H17 cells, ThPOK continues to function as a master regulator of the helper T cell lineage and continues to suppress the CTL program. Consistent with that, naive *Thpok*-GFP donor spleen cells that differentiated into T_H17 effector cells in the intestine of the *Rag1*$^{-/-}$ recipient mice remained GFP+ (**Fig. 4a**), which indicated that their gene-expression profile was still regulated by the ThPOK. To examine the effect of the loss of ThPOK expression on the gene-expression pattern of CD4+ effector cells, we analyzed gene microarrays generated from RNA isolated from sorted ThPOK+ or ThPOK− SP and DP effector cells from normal unmanipulated *Thpok*-GFP reporter mice[19] (**Fig. 4b**) and from *Rag1*$^{-/-}$ recipients of naive *Thpok*-GFP CD4+ SP donor cells (**Fig. 4c** and **Supplementary Fig. 3a**). Notably, we found that DP and SP ThPOK− CD4+ T cells had a unique but similar gene-expression pattern that differed from that of SP ThPOK+ CD4+ cells, although in the transfer experiments, the donor cells originated from the same pool of naive lymphocytes and differentiated in the same host environment (**Fig. 4b**,**c** and **Supplementary Fig. 3a**). As expected, many of the ThPOK+ CD4+ SP cells isolated from the intestine had a distinct T_H17 gene-expression pattern, whereas expression of those genes was barely detectable in the ThPOK− and DP subsets (**Fig. 4b**–**d**). Furthermore, RT-PCR analysis of genes characteristic of T_H17 cells, including those encoding the cytokines IL-17A, IL-17F and IL-22, the cytokine receptor IL-23R and the T_H17 hallmark nuclear transcription factor RORγt, confirmed that ThPOK− CD4+ effector cells were distinct from T_H17 cells (**Fig. 4e**,**f** and **Supplementary Fig. 3b**). Moreover, analysis of genes characteristic of other CD4+ helper T cell types, such as T_H1 and T_H2 cells, demonstrated that the gene-expression pattern of CD4+ SP lymphocytes and DP lymphocytes that had lost ThPOK expression did not resemble the patterns of the known helper T cell effector subsets (**Supplementary Fig. 3c**).

In contrast, in addition to reexpressing CD8α, ThPOK− CD4+ effector cells also expressed many other genes typically associated with the CD8+ CTL program. This included the expression of various genes encoding cytolytic proteins, such as several granzymes and perforin, as well as interferon-γ (IFN-γ) and several receptors expressed by natural killer cells and mature CD8+ CTLs (**Fig. 4b**,**c**,**g**,**h** and **Supplementary Fig. 3d**,**e**). Of particular note was their expression of the cytotoxicity-related, MHC class I–restricted, T cell–associated molecule CRTAM and the CD2 family member CD244 (2B4), both known to promote the cytolytic function and IFN-γ production of CD8+ T cells[25–27].

The reciprocal expression in mature CD4+ effector cells of either ThPOK or CTL signature genes (**Fig. 4i**) confirmed the hypothesis that ThPOK continuously suppressed the CTL program in conventional helper T cell cells but also demonstrated that the differentiation

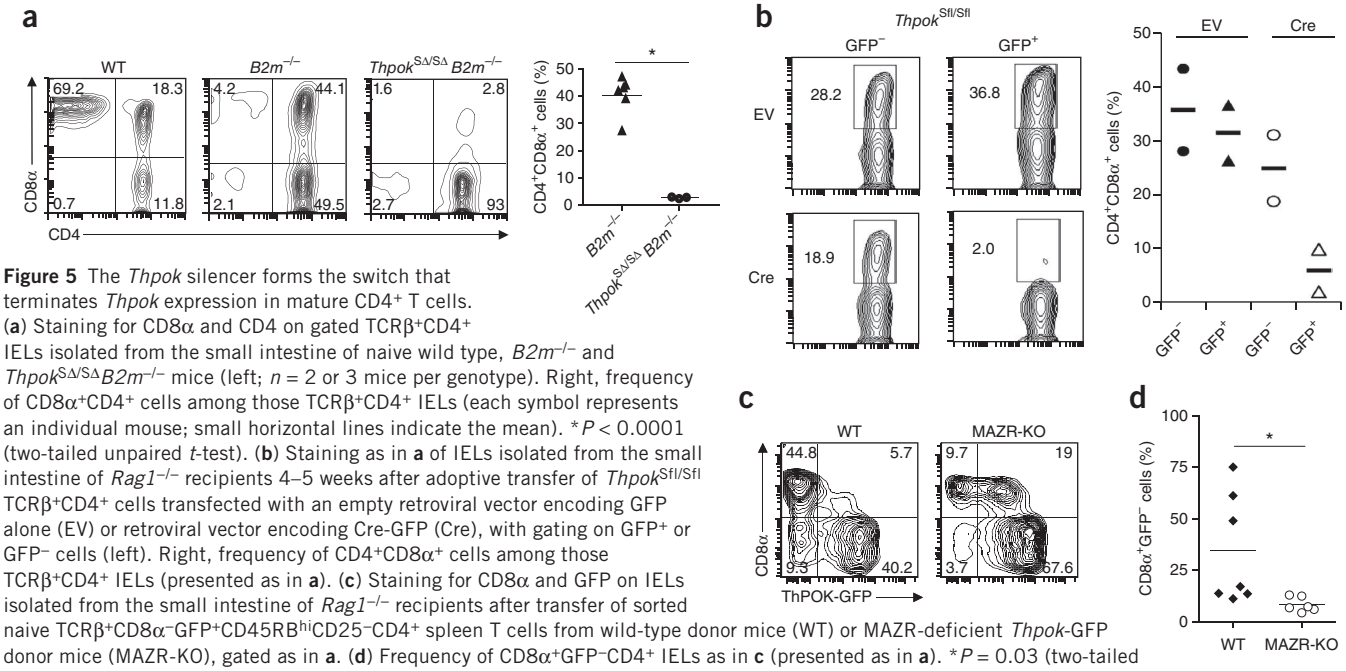

Figure 5 The *Thpok* silencer forms the switch that terminates *Thpok* expression in mature CD4+ T cells. (**a**) Staining for CD8α and CD4 on gated TCRβ+CD4+ IELs isolated from the small intestine of naive wild type, *B2m*$^{-/-}$ and *Thpok*$^{SΔ/SΔ}$*B2m*$^{-/-}$ mice (left; *n* = 2 or 3 mice per genotype). Right, frequency of CD8α+CD4+ cells among those TCRβ+CD4+ IELs (each symbol represents an individual mouse; small horizontal lines indicate the mean). *$P < 0.0001$ (two-tailed unpaired *t*-test). (**b**) Staining as in **a** of IELs isolated from the small intestine of *Rag1*$^{-/-}$ recipients 4–5 weeks after adoptive transfer of *Thpok*$^{Sfl/Sfl}$ TCRβ+CD4+ cells transfected with an empty retroviral vector encoding GFP alone (EV) or retroviral vector encoding Cre-GFP (Cre), with gating on GFP+ or GFP− cells (left). Right, frequency of CD4+CD8α+ cells among those TCRβ+CD4+ IELs (presented as in **a**). (**c**) Staining for CD8α and GFP on IELs isolated from the small intestine of *Rag1*$^{-/-}$ recipients after transfer of sorted naive TCRβ+CD8α−GFP+CD45RB^{hi}CD25−CD4+ spleen T cells from wild-type donor mice (WT) or MAZR-deficient *Thpok*-GFP donor mice (MAZR-KO), gated as in **a**. (**d**) Frequency of CD8α+GFP−CD4+ IELs as in **c** (presented as in **a**). *$P = 0.03$ (two-tailed unpaired *t*-test). Data are representative of three (**a**,**c**,**d**) or two (**b**) independent experiments.

of CD4+ CTL effector cells coincided with the post-thymic loss of ThPOK. In agreement with that, after enforced expression of ThPOK via retroviral transduction, CD4+ donor cells no longer differentiated into CTLs *in vivo* (**Fig. 4j**), whereas transfection of a construct encoding *Thpok* with a spontaneous mutation found in the helper-deficient mouse strain[4] did not prevent the differentiation of helper T cells into CD4+ CTLs (**Supplementary Fig. 3f**). These data indicated that the loss of ThPOK coincided with derepression of the CTL phenotype in mature CD4+ effector cells.

Thpok silencer derepression terminates *Thpok* expression

In thymocytes committed to the CD8+ CTL lineage, ThPOK expression is switched off at the transcriptional level by the *Thpok* silencer[22]. Consequently, in mice with germline deletion of this silencer (*Thpok*$^{SΔ/SΔ}$ mice), MHC class I–restricted thymocytes mature as CD4-expressing T cells in the periphery[22]. To determine if the *Thpok* silencer engages in a similar role to terminate *Thpok* expression in mature MHC class II–restricted precursors of CD4+ CTLs, we analyzed the peripheral maturation of CD4+ T cells in *Thpok*$^{SΔ/SΔ}$ mice. To eliminate the MHC class I–restricted CD4-expressing cells in these mice, we crossed the *Thpok*$^{SΔ/SΔ}$ mice with MHC class I–deficient (β_2-microglobulin-deficient (*B2m*$^{-/-}$)) mice, which abrogated the development of MHC class I–restricted CD4+CD8+ T cells. Notably, we found considerable enhancement of all CD4+ populations, including DP IELs, in *B2m*$^{-/-}$ mice, in the absence of mature CD8+ T cells (**Fig. 5a**); however, *Thpok*$^{SΔ/SΔ}$*B2m*$^{-/-}$ mice had considerably fewer mature MHC class II–restricted CD8α-expressing CD4+ T cells (**Fig. 5a**). These observations indicated that the *Thpok* silencer was a critical genomic switch in the process of the differentiation of CD4+ CTLs that turned off *Thpok* transcription in mature CD4+ T cells. We further confirmed that idea with CD4+ T cells in which a *lox*P-flanked *Thpok* silencer (*Thpok*$^{Sfl/Sfl}$) was deleted at the mature stage by transfection of the cells with a retroviral construct containing sequence encoding Cre linked to GFP. The transfer of transfected *Thpok*$^{Sfl/Sfl}$ CD4+ T cells (which expressed Cre (GFP+) and consequently had deletion of the *Thpok* silencer) into *Rag1*$^{-/-}$ recipient mice resulted in impaired accumulation of CD8α-expressing CD4+ CTLs in the intestine of the recipients (**Fig. 5b**), which further emphasized the critical role of the *Thpok* silencer in terminating *Thpok* expression as part of the CTL-differentiation process of CD4+ effector cells. In contrast to ThPOK, which suppresses the repressive activity of the *Thpok* silencer, the zinc-finger transcription factor MAZR is known to activate the *Thpok* silencer[28], which results in MAZR-induced negative regulation of *Thpok* transcription. Consistent with the participation of MAZR in reactivation of the *Thpok* silencer in mature CD4 T cells, MAZR-deficient CD4+ donor cells transferred into *Rag1*$^{-/-}$ recipient mice formed fewer CD8α-expressing CD4+ CTLs than did their wild-type counterparts in the intestine of the recipients (**Fig. 5c**,**d**). These results indicated that transcriptional regulation of *Thpok* was key for control of the helper T cell phenotype of mature CD4+ T cells and that reactivation of its silencer led to termination of the helper T cell program and, conversely, to the functional differentiation of MHC class II–restricted CD4+ CTLs.

CD4+ CTL differentiation is driven *in vivo* by antigen

The loss of ThPOK observed in progeny of naive ThPOK+ donor cells suggested that induction of the CTL program in mature CD4+ T cells coincided with an activation or maturation process. The reexpression of CD8α as well as the cytolytic functional differentiation of mature CD4+ T cells was reminiscent of the cytotoxic-lineage differentiation of positively selected CD8+ SP thymocytes mediated by IL-7 (ref. 29). Despite that, however, we found significantly more CD8α-expressing CD4+ CTLs in mice deficient in the receptor for IL-7 than in wild-type mice (**Fig. 6a**), which indicated that the *in vivo* differentiation of mature CD4+ CTLs was not an IL-7-driven process. Similar to other effector cells in the intestine, DP cells were not present in germ-free mice (**Supplementary Fig. 4a**) and seemed to be present in normal numbers in germ-free mice reconstituted with specific pathogen–free microorganisms (**Supplementary Fig. 4b**), which indicated that some microbial factors directly or indirectly promoted the accumulation of CD4+ CTLs in the intestine. In contrast, unlike classic CD4+ T_H17 effector cells, they did not increase in number in the intestine of

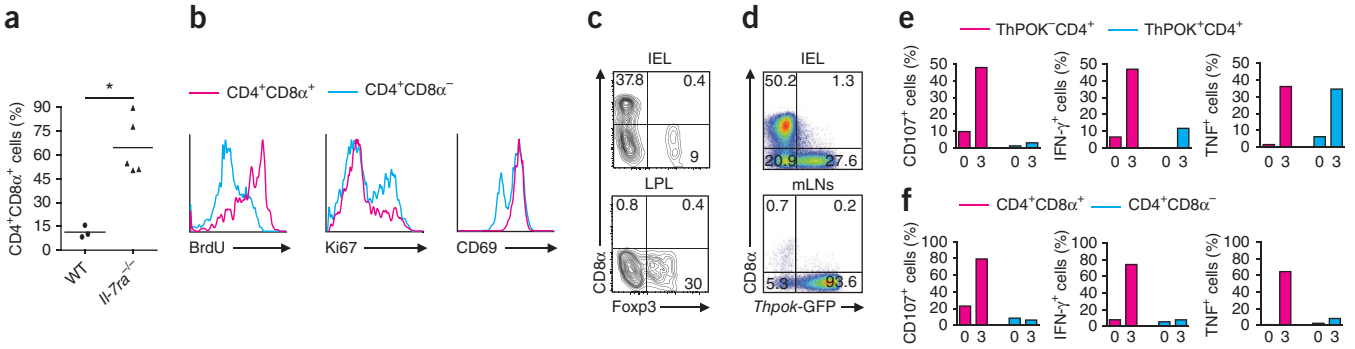

Figure 6 The ThPOK loss and reprogramming of CD4+ CTLs is an antigen-driven process *in vivo*. (**a**) Frequency of CD4+CD8α+ T cells among gated CD45+TCRβ+CD4+CD8β− IELs from wild-type mice or mice deficient in the receptor for IL-7 (*Il7ra*−/−). Each symbol represents an individual mouse; small horizontal lines indicate the mean. **P* = 0.03 (nonparametric two-tailed Mann-Whitney test). (**b**) BrdU staining of IELs from the small intestine of naive wild type mice after intraperitoneal injection of 1 mg BrdU, followed by 6 d of BrdU (0.8 mg/ml) in the drinking water (left), and staining for Ki67 (middle) and CD69 (right) of CD8α+ or CD8α− gated CD45+TCRβ+CD8β−CD4+ IELs from naive wild-type mice. (**c**) Staining for CD8α and Foxp3 in gated CD45+TCRβ+CD8β−CD4+ IELs (top) and lymphocytes from the lamia propria (bottom) of OT-II *Rag1*−/− mice (*n* = 4) after 4 weeks on an OVA-containing diet. (**d**) Frequency of CD8α+ and GFP+ cells among CD45+TCRβ+ CD4+ OT-II *Thpok*-GFP IELs isolated from *Rag1*−/− recipient mice after 4 weeks on an OVA-containing diet. (**e**) Frequency of CD107+, IFN-γ+ and TNF+ cells among OT-II *Thpok*-GFP ThPOK− or ThPOK+ CD45+TCRβ+ CD4+ IELs as in **d**, analyzed at day 0 without IL-15 exposure (0) or after 3 d of *in vitro* culture with recombinant IL-15 (3) before restimulation with OVA(323–339). (**f**) Frequency of CD107+, IFN-γ+ and TNF+ cells among wild-type CD8α+ and CD8α− CD45+TCRβ+CD8β−CD4+ polyclonal IELs cultured without or with IL-15 as in **e** before restimulation with antibody to TCR-β. Data are representative of two (**a**–**c**), five (**d**) or three (**e**,**f**) independent experiments.

germ-free mice monocolonized with segmented filamentous bacteria alone[30] (**Supplementary Fig. 4b**) or in response to an infection with *Citrobacter rodentium,* a pathogen known to induce a strong T_H17 response in the large intestine (**Supplementary Fig. 4c**), whereas they were present in normal numbers in the intestine of mice deficient in the adaptor MyD88. These observations indicated that the microbial conditions required for the steady-state accumulation of CD4+ CTLs in the intestine were probably not established by a single microorganism and did not depend on IL-1 signals or signals induced by Toll-like receptors (**Supplementary Fig. 4d**). Published reports have indicated that CD4+ CTLs are antigen-experienced cells that differentiate in response to repeated activation signals[10,12]. In agreement with that, DP cells with a CTL phenotype isolated from *Rag1*−/− recipients of naive spleen CD4+ SP T cells also showed evidence of strong or repeated activation and considerable uptake of the thymidine analog BrdU but only weak staining for the active cell-cycle marker Ki67 (**Fig. 6b**), which indicated that they were resting cells that had previously been activated and had previously intensely proliferated. In addition, CD4+ CTLs also had higher expression of the activation marker CD69 than did CD4+ SP effector helper T cells (**Fig. 6b**), which suggested that the activation process that coincided with the loss of ThPOK expression and derepression of the CTL program also coincided with those CD4+ effector cells that received strong or repeated activation signals. To directly investigate that, we analyzed the effector differentiation of monoclonal OT-II CD4+ T cells (which have transgenic expression of a TCR specific for ovalbumin amino acids 323–339 (OVA(323–339)) in response to continuous activation *in vivo* with their cognate peptide OVA(323–339) presented by I-A^b. T cells migrate as effector cells only to peripheral tissues, and in agreement with that, in the absence of OVA antigen, very few OT-II CD4+ T cells accumulated in the intestine of OT-II mice. Similarly, after transfer of naive OT-II CD4+ T cells into *Rag1*−/− recipient mice, only a limited number of the donor cells migrated to the intestine of the recipient mice in the absence of OVA antigen (data not shown). In contrast, after mice were fed an OVA-containing diet for at least 4 weeks, a large number of activated OT-II CD4+ cells accumulated in the intestine of the OT-II mice and the *Rag1*−/− recipients, and, notably, many reexpressed CD8α (**Fig. 6c**), whereas those that remained SP had a tendency to express the transcription factor Foxp3 (**Fig. 6c**). To determine if the derepression of CD8α expression on the responder OT-II CD4+ T cells coincided with the loss of ThPOK and their differentiation into CTLs, we isolated cells from OT-II *Thpok*-GFP reporter mice, transferred those naive donor cells into OVA-fed *Rag1*−/− recipient mice and analyzed the phenotype and function of the OVA-responding cells that accumulated as effector cells in the intestine of the hosts. As expected, many OT-II *Thpok*-GFP DP cells accumulated in the small intestine but, notably, they were all GFP− (**Fig. 6d**), which indicated that they had lost ThPOK expression. Furthermore, in addition to the reexpression of CD8α, OT-II GFP− effector cells also newly induced the expression of typical cytolytic markers, such as 2B4 and granzyme B (**Supplementary Fig. 5a**). Notably, they had an antigen-specific cytolytic response when restimulated *in vitro* with the OT-II TCR–specific peptide OVA(323–339) (**Fig. 6e**) but not when restimulated with the MHC class I–restricted OVA peptide SIINFEKL (amino acids 257–264; **Supplementary Fig. 5b**).

Despite their functional potential, OT-II ThPOK− CD4+ CTLs remained immunologically quiescent *in vivo* even in the continuous presence of their cognate antigen in the diet. That finding was similar to results obtained with MHC class I–restricted CD8αβ+ CTLs, which also seem to be inactive under steady-state conditions[31]. After challenge with excess amounts of IL-15, however, as in active celiac disease[18,32], CD8+ CTLs become pathogenic killer cells[33,34] that also considerably upregulate secretion of the inflammatory cytokines IFN-γ and tumor-necrosis factor (TNF)[18,35,36]. To determine if CD4+ CTLs might be as responsive to IL-15 as CD8+ CTLs, we restimulated the diet-induced OT-II ThPOK− CD4+ CTLs *in vitro* in the context of IL-15. As expected, the addition of IL-15 resulted in higher CD8α expression[37]; however, IL-15 alone or together with the irrelevant MHC class I–restricted peptide SIINFEKL had no effect on the functional differentiation or maturation of the MHC class II–restricted OT-II CD4+ CTLs (**Supplementary Fig. 5b**). In contrast, the addition of IL-15 resulted in considerably enhanced immune responses of OVA(323–339)-stimulated OT-II ThPOK− CD4+ CTLs, as measured by the substantal upregulation of CD107a expression and the

much greater production of IFN-γ and TNF (**Fig. 6e**). These data therefore demonstrated that antigen-induced CD4$^+$ CTLs generated in the absence of inflammation were poised to exert potent effector functions when reactivated by their cognate antigen in the context of the inflammatory cytokine IL-15. We confirmed those data with polyclonal ThPOK$^-$ CD4$^+$ CTLs isolated from the intestine of normal lymphocyte-sufficient mice and showed that unmanipulated CD4$^+$ CTLs analyzed *ex vivo* also had the potential in response to polyclonal activation and to increase their cytolytic and inflammatory immune responses when exposed to IL-15. Although IL-15 alone supported short-term survival of wild-type CD4$^+$ CTLs and CD8αβ$^+$ CTLs, this cytokine alone did not induce cytolytic effector functions in these polyclonal cells (**Supplementary Fig. 5c**). In contrast, similar to results obtained with the dietary antigen–induced OT-II ThPOK$^-$ CD4$^+$ CTLs or normal CD8αβ$^+$ CTLs (**Supplementary Fig. 5d**), the addition of IL-15 also resulted in considerably enhanced cytolytic and inflammatory functions of wild-type polyclonal TCR-stimulated CD4$^+$ CTLs, whereas it had only a negligible effect on ThPOK$^+$ CD4$^+$ helper T cells (**Fig. 6f**). These data emphasizes the likely pathogenic potential of antigen-induced ThPOK$^-$ CD4$^+$ CTLs, which were poised to kill under conditions in which IL-15 was abundant.

DISCUSSION

The results reported here have indicated an unexpectedly large degree of plasticity for CD4$^+$ helper T cells and have demonstrated that in response to chronic or strong stimulation with antigen, mature CD4$^+$ cells were able to terminate their expression of the helper T cell master regulator ThPOK (the signature transcription factor of the CD4 lineage) and differentiate into functional CTLs. The identification of additional functional CD4$^+$ T lymphocyte subsets defined by signature transcription factors, such as T_H17 cells and Foxp3$^+$ regulatory T cells, has been an important advance in immunology research. Although cytotoxic activity of CD4$^+$ T cells has long been known, here we have shown that this was not simply a function of T_H1-like cells. Instead, here we identified a previously unknown type of mature, antigen-experienced CD4$^+$ T cell characterized by the activation-induced loss of ThPOK. These cells had the concomitant appearance of a cell-surface and functional phenotype that was distinct from that of conventional helper T cell subsets but resembled that of CD8$^+$ effector CTLs. Our data therefore indicate that the ThPOK-driven commitment to the CD4$^+$ helper T cell lineage is not fixed and that plasticity controlled at the level of ThPOK expression may lead to the functional differentiation of defined ThPOK$^-$ MHC class II–restricted CD4$^+$ CTLs, despite their initial ThPOK expression and commitment to the helper T cell lineage or the MHC class II restriction of their TCRs.

The differentiation of CD4$^+$ T lymphocytes into CTLs instead of their suppression and prevention from becoming inflammatory T_H1 or T_H17 cells makes teleological sense at epithelial interfaces, where rapid elimination of infected cells is important not only for resistance to the initial invasion of intracellular pathogens but also for limiting or preventing excessive infiltration of systemically activated cytokine secreting cells that could jeopardize the integrity of the mucosal barrier[38]. In addition, the cytotoxic function of the CD4$^+$ CTLs may also contribute to diminishing infection and inflammation by eliminating infected migratory dendritic cells that could further prime naive cells or mediate systemic spread of the pathogen.

The MHC class II restriction of CD4$^+$ CTLs might render these lymphocytes able to restrain viral infections tropic for MHC class II–positive target cells, including infected MHC class II–positive epithelial cells[39], Epstein-Barr virus–transformed B cells[40] or human immunodeficiency virus–infected human CD4$^+$ T cells[41]. Such T cells might also be critical for protection against viruses, including human immunodeficiency virus and cytomegalovirus, that have developed mechanisms to escape surveillance by MHC class I–restricted CD8$^+$ CTLs[40,42] or under conditions in which the control response of CD8$^+$ CTLs becomes exhausted, as is the case in many chronic infections. In addition, as with CD8αβ$^+$ CTLs, the responsiveness of CD4$^+$ CTLs to IL-15 might provide a means for promoting their antigen-specific or bystander protective ability against a variety of pathogens or for enhancing the efficacy of antitumor therapies involving adoptive cells.

It is also possible, however, that in inflammatory disorders that involve IL-15, such as celiac disease or virus-induced inflammatory conditions[32,43], CD4$^+$ CTLs may increasingly become pathogenic effector cells that harmfully destroy their target tissue and recruit other cell types of the immune system, thereby contributing to inflammatory pathogenesis. In the context of celiac disease, it has been difficult to connect the known pathogenic factors, including dietary antigens, association with MHC class II, IL-15 and TCR-dependent and TCR-independent killer cells[18,36]. Our finding that dietary antigen–induced, MHC class II–restricted CD4$^+$ CTLs became potent cytotoxic effector cells, producing large amounts of IFN-γ and TNF, when stimulated by their cognate antigen in the presence of IL-15 may form the long-sought 'missing link' that ties together the genetic and environmental factors involved in the pathogenesis of celiac disease.

In summary, the results presented here have identified CD4$^+$ CTLs as distinct CD4$^+$ effector cells defined by their post-thymic loss of ThPOK and the reciprocal functional differentiation of these MHC class II–restricted T lymphocytes into CTLs. Our data have also identified the *Thpok* silencer as the molecular switch that drives the unique post-thymic reprogramming of these CD4$^+$ effector cells. The unexpected plasticity of mature CD4$^+$ T cells in differentiating into CTLs expands the functional ability of MHC class II–restricted CD4$^+$ effector cells to also include direct protective functions and provides the immune system with an alternative protective mechanism, although the findings here have also demonstrated that these CD4$^+$ CTLs may be central in driving the immunological pathology associated with inflammatory MHC class II–restricted T cells.

CD4$^+$ effector cells with cytolytic functions have been known for a long time, and they have frequently been associated with beneficial as well as pathological immune responses. Nevertheless, progress in directly linking such cells to those events or targeting them as part of an immunological therapy has been extremely difficult mainly because they could not be separated from classic effector helper T cells. Therefore, the new insights reported here defining CD4$^+$ CTLs as a distinct subset of effector cells and identifying the mechanism that leads to the unique differentiation of these cells provide information to finally move forward the field of CD4$^+$ CTLs and to specifically target these cells to enhance protective immunity or to prevent or treat inflammatory diseases and immunological pathology.

METHODS

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

Accession codes. GEO: microarray data, GSE41257 and GSE42277.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

H.C., I.T., M.K., D.M. and M.M.H. conceived of the project; M.M.H., D.M. and F.v.W. generated the phenotypic and functional data; I.T., S.M., Y.N. and C.M. generated the data on fate mapping and deletion of the *Thpok* silencer and did the ChIP assays; R.S. transfected cells; Y.H. provided the data on IL-7R-deficient mice; B.S.R., M.D. and A.A. generated the gene arrays; G.K., F.L. and C.J.L. transferred cells and analyzed mice; J.-W.S. and D.M. infected mice with citrobacter; K.A. and K.H. reconstituted germ-free mice; S.S. generated the data on the role of MAZR; Y.P. analyzed *Myd88*$^{-/-}$ mice; P.W., D.M., F.v.W., B.S.R. and H.C. analyzed the gene-array data; T.N. and W.E. provided expertise; M.K. provided conceptual advice and helped with data analysis and writing of the manuscript; I.T. and H.C. generated concepts, designed experiments, analyzed data and wrote the manuscript; and all authors contributed to the writing of the manuscript and provided advice.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. C57BL/6 CD45.1$^+$ (Ly5.1$^+$) and CD45.2$^+$ (Ly5.2$^+$) mice, OT-II mice, *Rag1*$^{-/-}$ mice and mice deficient in the receptor for IL-7 (all on a C57BL/6 background) were from The Jackson Laboratories, and CD4-Cre and C57BL/6 mice were from Taconic Farms. Swiss-Webster and C57BL/6 germ-free and conventionally raised specific pathogen–free mice were from Taconic Farms or the University of North Carolina Gnotobiotic Core, respectively. Germ-free IQI mice were from Japan CLEA. Mice monocolonized with segmented filamentous bacteria have been described[44]. *Thpok*-GFP mice, *Thpok*$^{S\Delta/S\Delta}$ mice and FH-ThPOK mice have been described[19,22]. OT-II *Thpok*-GFP mice were generated by the breeding of OT-II mice with *Thpok*-GFP reporter mice. *Thpok*$^{S\Delta/S\Delta}$ mice were crossed with *B2m*$^{-/-}$ mice (The Jackson Laboratories) to obtain *Thpok*$^{S\Delta/S\Delta}$*B2m*$^{-/-}$ mice. *Thpok*$^{Sfl/Sfl}$ mice were generated in the Tanuichi laboratory (I.T., unpublished data). Sequences 826 base pairs in length that included the core *Thpok* silencer were flanked by two *lox*P sites in the *Thpok* allele. E8$_I$-deficient mice were provided by D. Littman. MAZR-deficient mice were generated as described[28] and were crossed with *Thpok*-GFP mice. To generate mice with transgenic expression of Cre specifically in the CD4 lineage (ThPOK-Cre mice), we inserted a 562–base pair fragment of the *Thpok* silencer into a mini-transgene containing the minimal *Cd4* enhancer-promoter. To target transgene expression, we inserted sequence encoding an internal ribosomal entry site and human CD2 downstream of the cDNA cassette encoding Cre. Mice were maintained at the La Jolla Institute for Allergy and Immunology vivarium under specific pathogen–free conditions, and 'sentinel mice' from the *Rag1*$^{-/-}$ mouse colony were tested to be negative for *Helicobacter* species and *C. rodentium*. Animal care and experimentation were consistent with the guidelines of the US National Institutes of Health and were approved by the institutional Animal Care and Use Committees of the La Jolla Institute for Allergy and Immunology, the RIKEN Research Center for Allergy and Immunology, the Department of Immunology, Graduate School of Medicine, University of Tokyo and the Medical University of Vienna.

Antibodies and flow cytometry. The following antibodies were used: antibody to CD4 (anti-CD4; RM4-5), anti-CD25 (PC61), anti-CD44 (IM7), anti-CD69 (H1.2F3), anti-CD107a (1D4B), anti-CD107b (ABL-93), anti-V$_\beta$5.1 and anti-V$_\beta$5.2 (MR9-4), anti-CD244.2 (2B4) anti-Ki67 (B56) and anti-IFN-γ (XMG1.2; all from BD Biosciences); anti-TCRβ (H57-597), anti-TCRγδ (eBioGL3), anti-FoxP3 (FJK-16s) and anti-TNF (MP6-XT22; all from eBioscience); anti-CD8α (53-6.7), anti-CD8β (YTS156.7.7), anti-CD45.2 (104), anti-V$_\alpha$2 (B20.1) and anti-IL17A (TC11-18H10.1; all from Biolegend); and anti-CD8α (5H10) and anti–granzyme B (GB11; both from Invitrogen). Those antibodies were conjugated to the following fluorescent dyes: fluorescein isothiocyanate, phycoerythrin, peridinin chlorophyll protein–cyanine 5.5, allophycocyanin, allophycocyanin–efluor 780, Alexa Fluor 700, Pacific blue, phycoerythrin-indotricarbocyanine, phycoerythrin–Texas Red and Horizon V500. Antibody to mouse CD16-CD32 used for blocking Fc receptors was produced in the Cheroutre laboratory. In some experiments, a LIVE/DEAD Fixable Yellow Dead Cell Stain Kit (Invitogen) was used for analysis of only live cell events. Flow cytometry data were acquired on an LSR II (Becton Dickinson) and were analyzed with FlowJo software (TreeStar). Doublets were excluded by forward-scatter area versus forward-scatter width and side-scatter area versus side-scatter width. A Foxp3 Mouse Regulatory T cell Staining Kit (eBioscience) was used for intracellular staining of Foxp3 and granzyme B. For flow cytometry cytokine-secreting cells, cell populations were first stained with antibodies to cell surface markers, followed by permeabilization in Fix/Perm buffer and intracellular staining in Perm/Wash buffer (BD Pharmingen).

Preparation of IELs and lamina propria lymphocytes. IELs and lamina propria lymphocytes were isolated as described[45].

Culture of IELs. IELs enriched by anti-CD45 microbeads (Miltenyi Biotec) or sorted on a FACSAria were cultured in RPMI medium in the presence of recombinant mouse IL-2 (BD Biosciences) and recombinant human IL-15 (R&D Systems) at 37 °C with 5% CO_2.

Cohousing and microbiota reconstitution. Cohousing and microbiota reconstitutions were done as described[30].

Bacterial infection of mice. Wild-type *C. rodentium* strain DBS100 rendered resistant to chloramphenicol was obtained and used in all studies[46]. Bacteria were grown overnight in 3 ml Luria-Bertani broth with shaking at 37 °C, and bacterial titers were measured after each experiment by serial dilution. Mice were infected by oral gavage with 0.5×10^9 to 1.5×10^9 colony-forming units of *C. rodentium* (depending on experiment settings) and were killed at 14 d after infection.

Microarray and quantitative RT–PCR. RNA was prepared from sorted IEL populations with a FACSAria (Becton Dickinson). For microarray analysis, RNA was labeled and hybridized to GeneChip Mouse Genome 430 2.0 arrays according to Affymetrix protocols. Data were analyzed in GeneSpring GX10. RNA samples extracted with TRIZol (Invitrogen) with an iScript cDNA Synthesis kit (BIO-RAD) were used for cDNA synthesis. Real-time RT-PCR was done with the Roche 480 real-time PCR system. Values were normalized by the amount of the gene encoding the ribosomal protein L32 in each sample. Primer sequences are listed below. For Illumina microarray analysis, RNA was labeled and hybridized to Illumina MouseRef-8 v2.0 Expression BeadChips array according to the Illumina protocols. Data were analyzed with GeneSpring gene-expression analysis software (version 12.2). The heat maps were generated with the heatmap.2 function of R language. The inputs are $\log_2$-transformed, normalized microarray probe-set intensities. For genes with multiple probe sets on the array, average intensities were used to produce the heat map. Normalized values were used for unsupervised analysis with GeneSpring and analysis of gene ontology with IPA software (ingenuity pathway analysis). This analysis showed that the main changes were related to genes encoding molecules involved in T cell differentiation and activation. On the basis of those data, a supervised analysis of clusters of genes (or families) that most represented differences between the groups was made and presented. The full data sets were submitted to the GeoArchive database.

Adoptive-transfer model. Sorted naive (CD4$^+$CD45RB^{hi}) T cells (5×10^5) were transferred into *Rag1*$^{-/-}$ mice, as described[45]. The purity of sorted naive cells was >99.7% and was usually >99.9%. At 4–8 weeks after transfer, donor cells were isolated from various tissues of the *Rag1*$^{-/-}$ recipient mice and analyzed.

Generation of dietary antigen–induced OT-II CD4$^+$ IELs. For isolation of naive OT-II *Thpok*-GFP$^+$ CD4$^+$ cells, splenocyte samples were enriched for CD4$^+$ cells by positive selection with anti-CD4 microbeads according to the manufacturer's instructions (Miltenyi Biotec), followed by sorting for CD44^{lo} CD62L^{hi}V$_\alpha$2$^+$V$_\beta$5$^+$*Thpok*-GFP$^+$ CD4$^+$ cells with a FACSAria. Naive OT-II *Thpok*-GFP$^+$ CD4$^+$ cells (1×10^6) were transferred into the *Rag1*$^{-/-}$ recipients by the retro-orbital route. For the activation of antigen-specific OT-II T cells, those *Rag1*$^{-/-}$ recipients were fed an ovalbumin-containing diet for 4 weeks.

ChIP and tiling array. ChIP plus micrarray was done as described[19]. Chromatin DNA isolated from CD4$^+$ SP thymocytes (10×10^6) from FH-ThPOK mice was immunoprecipitated with anti-Flag (M2; Sigma-Aldrich), followed by two rounds of amplification by ligation-mediated PCR according to the manufacturer's protocol (Agilent). Custom microarrays generated by Agilent that tiled through the mouse *Cd8* locus by means of 60-nucleotide probes were used. Probe hybridization and scanning of oligonucleotide array data were done according to manufacturer's protocol (Agilent). Feature Extraction software and ChIP Analytics software (Agilent) were used for data analysis.

ChIP. Cells were fixed for 10 min with a 10% formaldehyde solution, followed by cell lysis and sonication to solubilize and shear crosslinked chromatin DNA. The resulting whole-cell extracts were incubated with anti–mouse immunoglobulin beads or anti–rabbit immunoglobulin beads (Invitrogen) and the appropriate antibody, then samples were washed and bound complexes were eluted from the beads. The primers used were as follows: E8$_I$ forward, 5′-CAATGCGAATGTGACTCAAG-3′, and E8$_I$ reverse, 5′-TAATGCGGTGTGATCAGTATG-3′; *Thpok* silencer

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forward, 5′-TGGTTTCGAGACTGGCTGGT-3′, and *Thpok* silencer reverse, 5′-GACCGAGGAGCTGCTTTCAG-3′.

Retroviral transduction of CD4 T cells. Primary $CD4^+$ T cells were transduced with retroviral vectors containing *Thpok, Thpok* with the spontaneous mutation found in the helper-deficient mouse strain, or sequence encoding Cre as described[47]. *Thpok* vectors were a gift from D. Kappes. Transfected cells were purified with a FACSAria, and 1×10^6 to 2×10^6 cells were transferred into $Rag1^{-/-}$ mice. Then, 4–5 weeks after transfer, tissues were recovered from recipient mice for flow cytometry.

Primers for quantitative PCR. These primers were as follows: GATA-3 forward, 5′-AGGATGTCCCTGCTCTCCTT-3′, and GATA-3 reverse, 5′-GCCT GCGGACTCTACCATAA-3′; ThPOK forward, 5′-ATGGGATTCCAAT CAGGTCA-3′, ThPOK reverse, 5′-TTCTTCCTACACCCTGTGCC-3′; T-bet forward, 5′-ATCCTGTAATGGCTTGTGGG-3′, and T-bet reverse, 5′-TC AACCAGCACCAGACAGAG-3′; CRTAM forward, 5′-TTAGAGTGAGC GTTTGGC CT-3′, and CRTAM reverse, 5′-GGGAGTCCTCAGTTTGCT GT-3′; RORγt forward, 5′-CCGCTGAGAGGGCTTCAC-3′, and RORγt reverse, 5′-TGCAGGAGTAGGCCACATTACA-3′; L32 forward, 5′-GAAA CTGGCGGAAACCCA-3′, and L32 reverse, 5′-GGATCTGGCCCTT GAACCTT-3′; IL-23R forward, 5′-TTCAGATGGGCATGAATGTTT CT-3′, and IL23R reverse, 5′-CCAAATCCGAGCTGTTGTTCTAT-3′; IL17A-forward, 5′-TGAGAGCTGCCCCTTCACTT-3′, and IL17A reverse, 5′-ACG CAGGTGCAGCCCA-3′; IL-22-forward, 5′-CAGGAGGTGGTACC TTTCCTGA-3′, and IL-22 reverse, 5′- TCTGGTCGTCACCGCTGAT-3′; IL-2-forward, 5′-CCTGAGCAGGATGGAGAATTACA-3′, and IL-2 reverse, 5′-TCCAGAACATGCCGCAGAG-3′; IL-4 forward, 5′-ACAGGAG AAGGGACGCCAT-3′, and IL-4 reverse, 5′-GAAGCCCTACAGACGAGC TCA-3′; IL-13 forward 5′-AGACCAGACTCCCCTGTGCA-3′, and IL-13 reverse, 5′-TGGGTCCTGTAGATGGCATTG-3′; granzyme A forward 5′-ATTCCT GAAGGAGGCTGTGAA-3′, and granzyme A reverse, 5′-GCAGGAGTCC TTTCCACCAC-3′; granzyme B forward 5′-GCCCACAACATCAA AGAACAG-3′, and granzyme B reverse, 5′-AACCAGCCACATAGCACA CAT-3′; CD8α forward, 5′-ACTGCAAGGAAGCAAGTGGT-3′, and CD8α reverse, 5′-CACCGCTAAAGGCAGTTCTC-3′; and IFN-γ forward, 5′-ATGAACGCTACACACTGCATC -3′, and IFN-γ reverse, 5′- CCATCCTTTT GCCAGTTCCTC -3′.

CD107 mobilization assay. The degranulation activity of cytolytic cells was measured as described[48]. Polyclonal IELs were enriched through the use of anti-CD45 microbeads (Miltenyi Biotec) and were then activated for 4 h at 37 °C and 5% CO_2 in 96-well U-bottomed plates with plate-bound antibody to TCRβ (2 μg/ml; H57-597; BD Biosciences) in the presence of anti-CD107a (1D4B; BD Biosciences) and brefeldin A. OT-II IELs were cultured for 6 h at 37 °C and 5% CO_2 in 96-well V-bottomed plates with antigen-presenting LB27.4 cells (mouse B cell lymphoma treated overnight with LPS) at a ratio of 5:1, in the presence of the MHC class II–specific peptide OVA(323–339) or the MHC class I–specific OVA peptide SIINFEKL peptide plus anti-CD107 and brefeldin A. In some cases, sorted IELs were cultured for 3 d in the presence of recombinant mouse IL2 (BD Biosciences) and recombinant human IL-15 (R&D Systems). After stimulation, the expression of CD107 and cytokines was assessed by flow cytometry.

***In vitro* cytotoxicity assay.** T cells crosslinked with anti-TCRβ were cultured for 6 h at 37 °C and 5% CO_2 with P815 mouse lymphoblast-like mastocytoma target cells, and cytotoxicity was measured with the CytoTox 96 Non-Radioactive cytotoxicity assay kit (Promega). The percentage of cytotoxicity was calculated on the basis of the release of lactate dehydrogenase. In some cases, cultured IELs were used.

Statistical analysis. Comparisons between groups used the Student's *t* test assuming two-tailed distributions unless otherwise stated, with an alpha value of 0.01–0.05. In some samples A nonparametric two-tailed Mann-Whitney test or analysis of variance (ANOVA) and Bonferroni post-test were used.

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