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Transcriptional Reprogramming of Mature CD4⁺ T helper Cells generates distinct MHC class II-restricted Cytotoxic T Lymphocytes

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H. C., I. T., M.K., D. M. and M. M. H. conceived 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 fate mapping- and *Thpok* silencer deletion data and performed the ChIP assays. R.S. performed the transfections. Y.H. provided the IL-7R KO data. B. S. R., M. D. and A. A. generated the gene arrays. G. K, F. L. and C. L. performed transfers and analyzed mice. J-W. S and D.M. performed the Citrobacter infections. K. A. and K. H. reconstituted germ-free mice. S. S. generated the data related to the role of MAZR. Y. P. analyzed the *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 the writing of the manuscript. I. T. and H. C. generated the concepts, designed experiments, analyzed data and wrote the manuscript with all authors contributing to the writing and providing advice.

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Abstract

TCR $\alpha\beta$ thymocytes differentiate to either CD8 $\alpha\beta$ cytotoxic T lymphocytes or CD4⁺ T helper cells. This functional dichotomy is controlled by key transcription factors, including the T helper master regulator, ThPOK, which suppresses the cytolytic program in MHC class II-restricted CD4⁺ thymocytes. ThPOK continues to repress CD8-lineage genes in mature CD4⁺ T cells, even as they differentiate to T helper effector subsets. Here we show that the T helper-fate was not fixed and that mature antigen-stimulated CD4⁺ T cells could terminate *Thpok* expression and reactivate CD8-lineage genes. This unexpected plasticity resulted in the post-thymic termination of the T helper-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 based on their roles in providing “help” to promote or dampen cellular and humoral immune responses. In contrast CD8 $\alpha\beta$ expressing cytotoxic T lymphocytes (CTLs) provide direct protective immunity by killing infected or transformed cells. The T helper (T_H)-program is initially induced during thymic development, where thymocytes expressing a major histocompatibility complex (MHC) class II-reactive T cell antigen receptor (TCR) develop into the CD4 T_H-lineage, whereas thymocytes with MHC class I specificity differentiate to the CD8 CTL-lineage. The functional programming, which coincides with, but does not depend on, the MHC restriction and CD4 or CD8 $\alpha\beta$ co-receptor expression, is controlled by the action and counteraction of key transcription factors. Together with Tox and GATA3, the T helper transcription factor, ThPOK (also known as cKrox; encoded by the gene, *Zbtb7b*, hereafter referred to as *Thpok*) first induces the CD4 T_H fate and prevents thymocytes from differentiating into CD8⁺ CTLs^{1–6}. The Runx family member, Runx3, has the opposite effect and terminates CD4 expression while promoting CTL-lineage differentiation^{7,8}. The CD8-CD4 lineage dichotomy persists in the periphery in mature T cells, where ThPOK continues to suppress the cytotoxic fate of MHC class II-restricted CD4⁺ T cells, even as they differentiate into T_H effector subsets, controlled by additional transcription factors⁶.

This lineage separation, however, is not all encompassing, and repeated reports have indicated the presence of CD4⁺ T cells with cytolytic functions in various species, including humans and rodents^{9–12}. At steady state, cytotoxic T cells, including CD4⁺ T cells, are enriched among the effector cells that reside as intraepithelial lymphocytes (IELs) in the intestine^{13–15}, whereas under inflammatory conditions, including viral infections, autoimmune disorders and in response to tumor antigens, many cytolytic CD4⁺ T cells expand in the blood and peripheral tissues^{9–12,16,17}. Although 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, convincing evidence directly linking them to specific aspects of the adaptive immune response has been difficult since they have been merely viewed as functional variants of the well-defined T_H1 subtype^{12,18}. The lack of a defined gene signature has also greatly

impaired progress on elucidating the biology of cytolytic CD4⁺ T cells and in designing clinical strategies that specifically target these cells in various diseases.

Here we provide compelling evidence indicating that cytotoxic CD4⁺ T cells represent a distinct subset of effector cells that could be defined by the absence of the master regulator, ThPOK, which maintains the T_H fate in all classical CD4⁺ T_H subsets by continuously suppressing the CD8 CTL-lineage program⁶. Nevertheless, we also show that the ThPOK-negative CD4⁺ CTLs originated from ThPOK-expressing progenitor cells that initially committed to the ThPOK-controlled T_H-lineage during thymic selection. These findings therefore challenge the notion that the T_H program in CD4⁺ T cells is fixed and show that mature CD4⁺ T cells can lose ThPOK expression post-thymically and functionally reprogram to become MHC class II-restricted CTLs. We also identify the *Thpok* silencer as the transcriptional switch that terminated *Thpok* transcription and by default drives the derepression of the CTL program in mature CD4⁺ effector cells. At steady state, CD4⁺ CTLs remained immune 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 to potent killer effector cells. Overall the data demonstrate that CD4⁺ CTLs are not a simple variant of classical ThPOK-controlled T_H1 cells, but that instead, they 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 CD8-CTL lineage gene expression program.

RESULTS

Not all mature CD4 T cells express ThPOK

The reported cytolytic activity of mature CD4⁺ T cells is inconsistent with the notion that ThPOK continuously suppresses the CTL program in all mature MHC class II-restricted CD4⁺ T cells⁶ 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 ThPOK-*green fluorescent protein (gfp)*-knock-in (ThPOK-*gfp*) reporter mice¹⁹. As expected, CD4⁺ ThPOK-*gfp* lymphocytes isolated from the spleen or mesenteric lymph node (mLN), which are mostly naïve T cells, were GFP-positive (GFP⁺), indicating that they all expressed ThPOK as is typical of mature CD4⁺ T_H-lineage cells (Fig. 1a). Conversely, all cells in the CD8⁺ fraction were GFP-negative (GFP⁻), consistent with the absence of ThPOK expression in CTL-lineage cells (Fig. 1a). Surprisingly, many of the CD4⁺ ThPOK-*gfp* effector T cells that at steady state accumulated in the intestine were GFP⁻, signifying that, like their CD8⁺ counterparts, they did not express ThPOK (Fig. 1b,c). Interestingly, the majority of the GFP⁻CD4⁺ cells resided in the subset of IELs that co-express CD8 α (without CD8 β)²⁰ (Fig. 1b-d). Consistent with the lack of ThPOK-mediated suppression, these CD8 α ⁺CD4⁺ double-positive (DP) cells also displayed functional features that were remarkably similar to those of mature CD8⁺ CTLs, including abundant expression of granzyme (Fig. 1e,f) and substantial levels of the activation-induced degranulation marker, CD107a, also known as lysosome-associated membrane protein 1 (LAMP-1), a glycoprotein present in the membrane of cytotoxic granules and exposed on the cell surface of activated cytolytic cells²¹ (Fig. 1g,h). The induction of CD107a by the DP subset was comparable to

that of typical CD8 TCR $\alpha\beta$ CTLs, whereas activated SP CD4⁺ IELs or T_H cells from the spleen did not induce this cytolytic marker (Fig. 1g,h). Moreover, activated DP CD4⁺ cells also effectively killed target cells *in vitro* as measured by the release of lactate dehydrogenase (LDH) upon target lysis (Fig. 1i and Supplementary Fig. 1a). In all, the data demonstrated that in normal mice, not all CD4⁺ effector cells expressed ThPOK and furthermore, that those CD4⁺ ThPOK-negative (ThPOK⁻) lymphocytes expressed CD8 α and displayed 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 T_H-lineage and is first expressed in the thymus where it counteracts Runx3 and suppresses the CTL fate of MHC class II-restricted thymocytes⁴⁻⁶. 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* gene transcription in MHC class I-specific CD8⁺ CTL lineage thymocytes is mediated by repressive factors, such as Runx proteins, that bind to the *Thpok* silencer (*S_{th}*) 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 that warrants continuous expression of *Thpok* (Fig. 2a). A fate marker that reports on 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. Based on this rationale, we designed a new Cre-transgenic mouse strain, referred to here as ThPOK-*Cre* mice, using a *Cre*-transgene construct in which we inserted the *Thpok* silencer into a *Cd4* enhancer/promoter locus that controls the *Cre* transgene expression (Fig. 2a). In contrast to the widely used CD4-*Cre* mice, which express *Cre* under the control of the *Cd4* enhancer/promoter starting at the CD4⁺CD8 $\alpha\beta$ ⁺DP thymocyte stage, in ThPOK-*Cre* mice, the *Thpok* silencer prevents *Cd4* enhancer/promoter activity in DP and CD8-lineage cells, resulting in exclusive Cre expression in CD4⁺ SP thymocytes committed to the T_H lineage (Fig. 2a). Therefore, when crossed to Rosa26-yellow fluorescent protein (YFP) reporter mice, only progeny derived from thymus-committed ThPOK-controlled CD4⁺ T_H lineage-committed thymocytes are marked by the expression of the YFP reporter after Cre mediated recombination (Fig. 2b,c). In these gene-reporter mice, SP CD8⁺ thymocytes and mature peripheral CD8⁺ T cells, including CD4⁻CD8⁺ mucosal T cells, did not express YFP, whereas, as expected, the majority of SP CD4⁺ LN T cells and IELs were YFP⁺. Notably, also CD8 α -expressing CD4⁺ IELs were YFP⁺, to a similar extent as the conventional CD4⁺ SP cells (Fig. 2b,c), indicating that they had induced ThPOK-dependent Cre expression, and therefore that ThPOK-negative mature CD4⁺ T cells must have derived from progenitor cells that expressed ThPOK at an earlier stage.

Mature ThPOK⁻ CD4 T cells lose ThPOK expression post-thymically

The notion that the mature ThPOK⁻ CD4⁺ cells previously expressed ThPOK, together with the observation that these cells mainly accumulate among effector cells, suggests that the loss of ThPOK expression might be the result of a post-thymic activation or maturation process that mature T cells undergo in the periphery. To test this, we adoptively transferred

highly purified ThPOK-expressing GFP⁺ naïve ThPOK-*gfp* CD4⁺ T cells to *Rag1*-deficient (*Rag1*^{-/-}) recipient mice. The lymphopenic conditions in this model induce strong proliferation and differentiation of the donor cells, which accumulate mainly as T_H17 cells (characterized by the expression of the transcription factor ROR γ t and the cytokine, IL-17) in the large intestine, and interestingly, also as DP effector cells especially in the small intestine of the *Rag1*^{-/-} hosts (Fig. 3a,b). Donor cells in the spleen or mLNs were GFP⁺ implying that they continued to express ThPOK (Fig. 3c). In contrast, many of the ThPOK-*gfp* CD4⁺ T cells that accumulated as effector cells in the intestine did not express detectable levels of GFP, indicating that they strongly reduced or completely lost ThPOK expression (Fig. 3c). Consistent with the observations in immune-replete mice, the loss of GFP expression was again most pronounced in those CD4⁺ cells that also re-induced CD8 α , although a small fraction of SP CD4⁺ IELs was also GFP⁻ (Fig. 3c,d). Serial transfer of those SP donor cells to a second set of *Rag1*^{-/-} recipients generated many more DP cells (Fig. 3e), indicating that the re-expression of CD8 α on mature CD4⁺ T cells follows the loss of ThPOK expression and further implies that the *Cd8a* locus in conventional CD4⁺ T_H cells might be constitutively suppressed by ThPOK. To analyze this, we performed chromatin immunoprecipitation (ChIP) combined with tiling arrays using cells from genetically engineered mice that express a Flag-hemagglutinin epitope-tagged ThPOK protein (FH-ThPOK) from the *Thpok* locus¹⁹. FH-ThPOK associated with the E8₁ enhancer element²⁴ in the *Cd8a* locus in SP CD4⁺ thymocytes (Fig. 3f). This result was also confirmed in mature SP CD4⁺ T cells using a ChIP assay (Supplementary Fig. 2a), suggesting that in mature CD4⁺ thymocytes and lymphocytes, ThPOK prevents CD8 α expression by direct suppression of the E8₁ enhancer element in the *Cd8a* locus, and also that the expression of CD8 α without CD8 β in mature ThPOK⁻ CD4⁺ T cells is driven by the de-repression of the E8₁ enhancer. In support of this finding, the percent of CD8 α -expressing CD4⁺ IELs and CD8 expression were both severely reduced in E8₁-deficient mice (Fig. 3g) or in the progeny of E8₁-deficient CD4 cells in *Rag1*^{-/-} recipient mice that previously received an equal number of naïve wild-type and E8₁-deficient CD4 donor cells (Fig. 3h and Supplementary Fig. 2b). Overall the data indicate that naïve ThPOK⁺ CD4⁺ T_H cells may lose expression of ThPOK in the periphery and consequently regain expression of ThPOK-suppressed genes such as *Cd8a*.

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, GATA3 for T_H2 and ROR γ t for T_H17 cells, ThPOK continues to function as a master regulator of the T_H-lineage and continues to suppress the CTL program. Consistent with this notion, in the adoptive transfer model, ThPOK-*gfp* donor cells that differentiated to T_H17 effector cells in the intestine of the recipient mice remained GFP⁺, indicating that their gene expression profile was still regulated by the ThPOK repressor (Fig. 4a). To examine the impact of the loss of ThPOK expression on the gene expression pattern of CD4⁺ effector cells, we analyzed gene microarrays generated from RNA isolated from either sorted ThPOK⁺ or ThPOK⁻ SP and DP effector cells from normal unmanipulated *Thpok*-GFP reporter mice¹⁹ (Fig. 4b) or from *Rag1*^{-/-} recipient mice (Fig. 4c and Supplementary Fig. 3a). Interestingly, the DP and SP ThPOK⁻ CD4⁺ T cells expressed a unique but similar gene expression pattern that clearly differed from that of the 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⁺ SP CD4⁺ cells isolated from the intestine displayed a clear T_H17 gene-expression pattern, whereas expression of these genes was barely detectable in the ThPOK⁻ and DP subset (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, ROR γ t (Fig. 4e,f and Supplementary Fig. 3b) confirmed that ThPOK⁻ CD4 effector cells were distinct from T_H17 cells. Furthermore, analysis of genes characteristic of other CD4⁺ T_H types, such as the T_H1 and T_H2 cells (Supplementary Fig. 3c), demonstrated that the gene expression pattern of SP and DP CD4⁺ lymphocytes that had lost ThPOK expression did not resemble the patterns expressed by the known T_H effector subsets.

In contrast, in addition to the re-expression of CD8 α , ThPOK⁻ CD4⁺ effector cells also expressed many other genes typically associated with the CD8 CTL-program, including 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 (NK) cells and mature CD8⁺ CTLs (Fig. 4b,c,g,h and Supplementary Fig. 3d,e). Of particular note was the expression of the cytotoxicity-related 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 cells²⁵⁻²⁷.

The reciprocal expression in mature CD4⁺ effector cells of either the transcription factor ThPOK or CTL signature genes (Fig. 4i), confirmed that ThPOK continuously suppresses the CTL program in conventional T_H cells but also demonstrated that the differentiation of CD4⁺ CTL effector cells coincides with the post-thymic loss of the T_H-lineage master regulator ThPOK. In agreement with this finding, enforced expression of ThPOK due to retroviral transduction, CD4⁺ donor cells no longer differentiated into CTLs *in vivo* (Fig. 4j), whereas transfection of a construct encoding a *Thpok* spontaneous mutation found in the helper-deficient mouse strain (HD-variant)⁴ did not prevent the CTL differentiation (Supplementary Fig. 3f). These data indicate that the loss of ThPOK coincided with the de-repression of the CTL phenotype in mature CD4⁺ effector cells.

***Thpok* silencer de-repression terminates *Thpok* expression**

In CD8 CTL-lineage committed thymocytes ThPOK expression is switched off at the transcriptional level by the *Thpok* silencer¹⁹. Consequently, in mice, which harbor a germline deletion of this silencer (*Thpok*^{S /S}), MHC class I-restricted thymocytes mature as CD4-expressing T cells in the periphery¹⁹. To investigate if the *Thpok* silencer engages in a similar role to terminate *Thpok* expression in mature MHC class II-restricted CD4⁺ CTL precursor cells, we analyzed peripheral CD4⁺ T cell maturation in mice in which the *Thpok* silencer had been deleted (*Thpok*^{S /S} mice). To eliminate the MHC class I-restricted CD4-expressing cells in these mice, we crossed the *Thpok*^{S /S} mice with MHC class I-deficient (*B2m*^{-/-}) mice, to abrogate the development of MHC class I-restricted DP T cells. Interestingly, whereas in wild-type *B2m*^{-/-} mice, in the absence of mature CD8⁺ T cells, all

CD4 populations are greatly enhanced, including the DP CD4⁺ IELs (Fig. 5a), in *Thpok^S/S* x *B2m^{-/-}* mice however, mature MHC class II-restricted CD8-expressing CD4⁺ T cells were severely reduced (Fig. 5a). These observations indicate that the *Thpok* silencer is a critical genomic switch in the process of CD4 CTL differentiation, which turns off *Thpok* transcription in mature CD4⁺ T cells. This notion was further confirmed using CD4⁺ T cells, in which the floxed *Thpok* silencer (*Thpok^{Sfl/Sfl}*) was deleted at the mature stage by transfecting the cells with a *Cre-gfp*-containing retroviral construct. Transfer of sorted GFP⁺ *Thpok^{Sfl/Sfl}* CD4⁺ T cells, which expressed Cre (GFP⁺) and consequently deleted the *Thpok* silencer, showed impaired accumulation of CD8-expressing CD4 CTL in the intestine of *Rag1^{-/-}* recipient mice, further underscoring the critical role of the *Thpok* silencer in terminating *Thpok* expression as part of the CTL differentiation process of CD4⁺ effector cells (Fig. 5b). 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* silencer²⁸, resulting in MAZR-induced negative regulation of *Thpok* gene transcription. Consistent with the participation of MAZR in the reactivation of the *Thpok* silencer in mature CD4 T cells, *Mazr^{-/-}* CD4⁺ donor cells formed fewer DP CTLs in the intestine of *Rag1^{-/-}* hosts (Fig. 5c,d). These results indicate that the transcriptional regulation of *Thpok* is key for the control of the T_H phenotype of mature CD4⁺ T cells, and that reactivation of its silencer leads to the termination of the T_H program and conversely to the functional differentiation of MHC class II-restricted CD4⁺ CTLs.

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

The loss of ThPOK observed in progeny of naïve ThPOK⁺ donor cells suggested that the induction of the CTL program in mature CD4⁺ T cells coincided with an activation or maturation process. In light of this, the re-expression of CD8α as well as the cytolytic functional differentiation of mature CD4⁺ T cells is reminiscent of the cytotoxic-lineage differentiation of positively selected CD8⁺ SP thymocytes mediated by IL-7²⁹. Despite this however, we found significant, and even increased numbers, of DP CD4⁺ T lymphocytes in *Il7ra^{-/-}* animals (Fig. 6a), indicating that the *in vivo* differentiation of mature CD4⁺ CTLs is not an IL-7 cytokine-driven process. Similar to other effector cells in the intestine, DP cells are absent in germ-free (GF) mice (Supplementary Fig. 4a), and appear in normal numbers in germ-free animals reconstituted with specific pathogen free (SPF) microorganisms (Supplemental Fig. 4b), indicating that some microbial factors directly or indirectly promote the accumulation of CD4⁺ CTLs in the intestine. In contrast, unlike classical CD4⁺ T_H17 effector cells, they did not expand in the intestine of germ-free mice mono-colonized with segmented filamentous bacteria (SFB) alone³⁰ (Supplemental 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 *Myd88^{-/-}* animals. These observations indicate that the microbial conditions required for the steady state accumulation of CD4⁺ CTLs in the intestine are not likely established by a single microorganism and do not depend on IL-1 or TLR-induced signals (Supplementary Fig. 4d). Published reports have indicated that CD4⁺ CTLs are antigen-experienced cells that differentiated in response to repeated activation signals^{10,12}. In agreement with this, in the adoptive transfer model, DP CD4⁺ donor cells, which displayed a CTL phenotype, also showed evidence of strong or repeated activation and

marked BrdU uptake, but only weak staining for the active cell cycle marker, Ki67 (Fig. 6b), indicating that they were resting cells that had been activated and intensely proliferated previously. In addition, the CD4⁺ CTLs also showed enhanced expression of CD69 as compared to the SP CD4⁺ T_H effector cells, suggesting that the activation process that coincided with the loss of ThPOK expression and de-repression of the CTL program also coincided with those CD4⁺ effector cells that received strong or repeated activation signals (Fig. 6b). To directly test this, we analyzed the effector differentiation of monoclonal OT-II TCR transgenic (OT-II) CD4⁺ T cells in response to a continuous activation *in vivo* with their cognate OVA peptide, (OVA₃₂₃₋₃₃₉) presented by I-A^b. T cells only migrate as effector cells to peripheral tissues and in agreement with this, in the absence of OVA, very few OT-II CD4⁺ T cells accumulated in the intestine of OT-II TCR transgenic animals. Similarly, upon transfer of naïve OT-II CD4⁺ T cells to *Rag1*^{-/-} recipients, only a limited number of these donor cells migrate to the intestine of the recipient mice in the absence of OVA antigen. In contrast, upon feeding an OVA-containing diet for at least 4 weeks, a large number of activated OT-II CD4⁺ cells accumulated in the intestine and interestingly, many re-expressed CD8α (Fig. 6c), whereas those that remained SP had a tendency to express Foxp3 (Fig. 6c). To investigate if the de-repression of CD8α on the responder OT-II CD4⁺ T cells coincided with the loss of ThPOK and the differentiation to CTLs, we transferred naïve donor cells isolated from OT-II ThPOK-*gfp* reporter mice to 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 DP OT-II ThPOK-*gfp* cells accumulated in the small intestine but more importantly they were all GFP⁻, indicating that they had lost ThPOK expression (Fig. 6d). Furthermore, in addition to the re-expression of CD8α, OT-II GFP⁻ effector cells also induced typical cytolytic markers, such as 2B4 and granzyme B (Supplementary Fig. 5a). More importantly, they displayed an antigen-specific cytolytic response when re-stimulated with the OT-II TCR specific OVA₃₂₃₋₃₃₉ peptide (Fig. 6e) but not with the MHC class I restricted OVA peptide (SIINFEKL) (Supplementary Fig. 5b).

Despite their functional potential ThPOK⁻ OT-II CD4⁺ CTLs remained immune quiescent *in vivo* even in the continuous presence of their cognate antigen in the diet. This finding is similar to the MHC class I-restricted CD8αβ CTLs, which also appear inactive under steady state conditions³¹. Upon challenge in excess amounts of IL-15, however, as in active celiac disease^{18,32}, CD8⁺ CTLs become pathogenic killer cells^{33,34} that also greatly upregulate secretion of the inflammatory cytokines, IFN-γ and tumor necrosis factor (TNF)^{18,35,36}. To investigate if CD4⁺ CTLs might be equally responsive to IL-15, we re-stimulated the diet-induced ThPOK⁻ OT-II CD4⁺ CTLs *in vitro* in the context of IL-15. As expected, IL-15 increased CD8α expression³⁷, 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, IL-15 greatly increased the immune responses of OVA₃₂₃₋₃₃₉-stimulated ThPOK⁻ OT-II CD4⁺ CTLs, as measured by the marked upregulation of CD107a expression and the significant increase in production of IFN-γ and TNF (Fig. 6e). These data therefore demonstrate that antigen-induced CD4⁺ CTLs, generated in the absence of inflammation, are poised to exert potent effector functions when re-activated by their cognate antigen in the

context of the inflammatory cytokine IL-15. We confirmed these data with polyclonal ThPOK⁻ CD4⁺ CTLs isolated from the intestine of normal lympho-replete animals and showed that unmanipulated CD4⁺ CTLs analyzed *ex vivo* also harbor the potential 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⁺ and CD8αβ⁺ CTLs, this cytokine alone did not induce cytolytic effector functions in these polyclonal cells (Supplementary Fig. 5c). On the contrary, similar to the diet antigen-induced ThPOK⁻ OT-II CD4⁺ CTLs, or normal CD8αβ⁺ CTLs (Supplementary Fig. 5d), IL-15 also strongly increased the cytolytic and inflammatory functions of wild-type TCR-stimulated CD4⁺ CTLs, whereas it had no effect on ThPOK⁺ CD4⁺ T_H cells (Fig. 6f). These data underscore the likely pathogenic role of antigen-induced ThPOK⁻ CD4⁺ CTLs, which are poised to kill under conditions where IL-15 is abundant.

DISCUSSION

The results reported here indicate an unexpected degree of plasticity for CD4⁺ T_H cells, and demonstrate that in response to chronic or strong antigen stimulation, mature CD4⁺ cells can terminate the expression of the T_H master regulator, ThPOK, and differentiate to 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 one of the most important recent advances in immunology research. Although cytotoxic activity carried out by CD4⁺ T cells has long been known, here we show that this is not simply a function of T_H1-like cells. Instead, here we identify a new type of mature, antigen-experienced CD4⁺ cells characterized by the activation-induced loss of the CD4 lineage signature transcription factor, ThPOK. These cells display the concomitant appearance of a cell surface- and functional phenotype that is distinct from that of conventional T_H subsets, but that strikingly resembles that of CD8⁺ CTL effector cells. The data therefore indicate that the ThPOK-driven thymic commitment to the CD4 T_H lineage is not fixed, and that plasticity controlled at the level of ThPOK expression may lead to the functional differentiation of defined ThPOK-negative MHC class II-restricted CD4⁺ CTLs, despite their initial ThPOK expression and T_H-lineage commitment or the MHC-class II restriction of their TCRs.

The differentiation of CD4⁺ T lymphocytes to CTL instead of suppressing them and prevent them from become inflammatory T_H1 and T_H17 cells, makes teleological sense at epithelial interfaces, where a rapid elimination of infected cells is important not only to resist the initial invasion of intracellular pathogens, but also to limit or prevent excessive infiltration of systemically activated cytokine secreting cells that could jeopardize the integrity of the mucosal barrier³⁸. In addition, the cytotoxic function of the CD4⁺ CTLs may also contribute to reducing the infection and inflammation by eliminating infected migratory DCs that could further prime naïve cells or mediate systemic spreading of the pathogen.

The MHC class II restriction of CD4⁺ CTLs might render these lymphocytes capable of restraining viral infections tropic for MHC class II⁺ target cells, including infected MHC class II⁺ epithelial cells³⁹, *Epstein-Barr virus* B cells⁴⁰ or *Human immunodeficiency virus* ((HIV)-infected human CD4⁺ T cells⁴¹. These T cells might also be critical for protection

against viruses, including HIV and *Cytomegalovirus*, that have developed mechanisms to escape surveillance by MHC class I-restricted CD8⁺ CTLs^{40,42} or under conditions where the CD8 CTL-control response becomes exhausted as is the case in many chronic infections. In addition, like CD8 $\alpha\beta$ CTLs, CD4⁺ CTL responsiveness to IL-15 might provide a means to promote their antigen-specific or bystander protective capacity against a variety of pathogens or for enhancing the efficacy of adoptive cellular anti-tumor therapies.

It is however also possible, in inflammatory disorders involving IL-15, such as celiac disease or virally induced inflammatory conditions^{32,43}, that CD4⁺ CTLs may increasingly become pathogenic effector cells that harmfully destroy their target tissue and recruit other immune cell types, thereby contributing to inflammatory pathogenesis. In the context of celiac disease, it has been difficult to connect the known pathogenic factors, including dietary antigens, MHC class II association, IL-15, and TCR-dependent and -independent killer cells^{18,36}. Our finding, that diet-antigen-induced MHC class II restricted CD4⁺ CTLs become 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 here identify CD4⁺ CTLs as distinct CD4⁺ effector cells, defined by their post-thymic loss of the T_H master transcription factor ThPOK and the reciprocal functional differentiation of these MHC class II-restricted T lymphocytes to CTLs. The data also identify the *Thpok*-silencer as the molecular switch, which drives the unique post-thymic reprogramming of these CD4⁺ effector cells. The unexpected plasticity of mature CD4⁺ T cells to differentiate 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 also demonstrate that these CD4⁺ CTLs may be central in driving the immune 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 these cells to these events or targeting them as part of immune therapies, has been extremely difficult mainly because they could not be separated from classical T_H effector 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 much needed information to finally move forward the field of CD4⁺ CTLs and to specifically target these cells to enhance protective immunity or prevent or treat inflammatory diseases and immune pathology.

METHODS

Mice

C57BL/6 CD45.1 (Ly5.1) and CD45.2 (Ly5.2), OT-II TCR-transgenic, *Rag1*^{-/-}, *Il7ra*^{-/-} and *Foxp3*^{eGFP} mice (all on a C57BL/6 background) were purchased from The Jackson Laboratories and CD4-*Cre* and C57BL/6 mice were obtained from Taconic Farms. Swiss-

Webster and C57BL/6 GF and conventionally raised specific pathogen-free (SPF) mice were purchased from Taconic Farms or from University of North Carolina Gnotobiotic Core, respectively. GF IQI mice were purchased from Japan CLEA Inc. Mice monoclonized with SFB were previously described⁴⁴. ThPOK-*gfp*, *Thpok^S/S* and ThPOK^{FH} were previously described^{19,22}. OT-II ThPOK-*gfp* mice were generated by breeding OT-II TCR transgenic mice with ThPOK-*gfp* reporter mice. *Thpok^S/S* mice were crossed with *B2m^{-/-}* mice (The Jackson Laboratories) to obtain *Thpok^S/S B2m^{-/-}* mice. Generation of *Thpok* silencer flox (*Thpok^{Sfl}*) allele in ES cells and *Thpok^{Sfl/Sfl}* mice will be described elsewhere. In brief, 826 bp sequences including the core *Thpok* silencer were flanked by two loxP site in the *Thpok^{Sfl}* allele. E8I-deficient mice were provided by D. Littman (New York University, NY). *Mazr^{-/-}* mice were generated as previously described²⁸ and crossed with ThPOK-*gfp* mice. To generate CD4-lineage specific *Cre*-transgenic mice (ThPOK-*Cre* mice), we inserted a 562 bp ThPOK silencer fragment into a mini transgene harboring the minimal CD4 enhancer-promoter. To target transgene expression, we inserted the IRES-hCD2 sequence downstream of the *Cre* cDNA cassette. Mice were maintained at the La Jolla Institute for Allergy and Immunology vivarium under SPF conditions and sentinel mice from the *Rag1^{-/-}* mice colony were tested to be negative for *Helicobacter spp.* and *C. rodentium*. Animal care and experimentation were consistent with the NIH guidelines 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 analysis

Fluorescent-dye-conjugated antibodies, including conjugates; FITC, PE, PercPcy5.5, APC, APCef-780, AF700, PB, PECy7, PE-Texas Red, V500 and antigens; CD4 (RM4-5), CD8 α (53-6.7), CD8 β (YTS156.7.7), CD25 (PC61.5), CD44 (IM7), CD45.2 (104), CD45 (30-F11), CD107 (1D4B), CD107b (ABL-93), V α 2 (B20.1), V β 5.1 and V β 5.2 (MR9-4), CD244.2 (2B4), granzyme B (GB11) and TCR β (H57-597) were from BD-PharMingen or eBioscience or BD-Biosciences or Biolegend or Invitrogen. Anti-mouse CD16/32 used for Fc receptor blocking was produced in our laboratory. In some experiments LIVE/DEAD Fixable Yellow dead cell stain kit (Invitrogen) was used to analyze only LIVE cell events. Flow cytometry data were acquired on a LSR-II instrument (Becton Dickinson) and analysed using FlowJo software package (Tri-Star). Doublets were excluded by Fsc-area versus Fsc-width and Ssc-area versus Ssc-width. Intracellular staining of Foxp3 and Granzyme B was conducted using Foxp3 Mouse Regulatory T cell Staining Kit (eBioscience). For flow cytometric analysis of cytokine-secreting cells, cell populations were first stained with antibodies against the indicated cell surface markers, followed by permeabilization in Fix/Perm buffer, and intracellular staining in Perm/Wash buffer (BD PharMingen).

Preparation of intraepithelial and lamina propria lymphocytes

Intraepithelial and lamina propria lymphocytes were isolated as previously described⁴⁵.

Culture of intraepithelial lymphocytes

Intraepithelial lymphocytes enriched by anti-CD45 microbeads (Miltenyi Biotec Inc.) or FACS Aria sorted, were cultured in RPMI medium in the presence of recombinant mouse IL-2 (BD Biosciences) and recombinant human IL-15 (R&D Systems Inc. at 37 °C with 5% CO₂.

Cohousing and Microbiota Reconstitution

Cohousing and microbiota reconstitutions were performed as described previously³⁰.

Bacterial infection of mice

Wild-type *C. rodentium* strain DBS100 rendered resistant to chloramphenicol was obtained and used in all studies⁴⁶. Bacteria were grown overnight in 3 ml Luria-Bertani (LB) broth with shaking at 37 °C and bacterial titers were determined after each experiment by serial dilution. Mice were regularly infected by oral gavage with $0.5\text{--}1.5 \times 10^9$ C.F.U. of *C. rodentium* (depending on experiment settings) and were sacrificed at 14 days post infection.

Microarray and qRT-PCR

RNA was prepared from sorted IEL populations using a FACS Aria cell sorter flow cytometer (Becton Dickinson). For microarray analysis, RNA was labeled and hybridized to GeneChip Mouse Genome 430 2.0 arrays according to the Affymetrix protocols. RNA was prepared from sorted IEL populations using a FACS Aria cell sorter flow cytometer (Becton Dickinson). Data were analyzed in GeneSpring GX10. Complementary DNAs (cDNAs) were synthesized from RNA samples extracted with TRIZol (Invitrogen) using an iScript cDNA Synthesis kit (BIO-RAD). Real-time RT-PCR was performed with the Roche 480 real-time PCR system. Values were normalized by the amount of L32 in each sample. Primer sequences are reported under **Primers for qPCR**. For Illumina microarray analysis, RNA was labeled and hybridized to Illumina MouseRef-8 v2.0 Expression BeadChips array according to the Illumina protocols. Data was analyzed in GeneSpring GS v12.2. The heat-maps were generated with the heatmap.2 function of R language. The inputs are base 2 log transformed normalized microarray probe set intensities. For genes with multiple probe sets on the array, the average intensities were used to produce heat-map. Normalized values were used for unsupervised analysis using gene spring and gene ontology in IPA (ingenuity pathway analysis). This analysis showed that major changes were related to T cell differentiation and activation genes. Based on these data a supervised analysis of clusters of genes (or families) that most represented differences between the groups was performed and shown. The full data sets were submitted to the GEOArchives.

Adoptive transfer model

5×10^5 sorted naïve (CD4⁺CD45RB^{hi}) T cells were transferred into *Rag1*^{-/-} mice, as previously described⁴⁵. Sorting purity of naive cells was higher than 99.7% and usually above 99.9%. 4 to 8 weeks after transfer, donor cells were isolated from various tissues of the *Rag1*^{-/-} recipient mice and analyzed as described.

Generation of diet antigen induced OT-II TCR transgenic CD4⁺ IELs

For naïve OT-II *Thpok*-GFP⁺ CD4⁺ isolation, splenocytes were enriched for CD4⁺ cells by positive selection using anti-CD4 microbeads according to the manufacturer's instructions (Miltenyi Biotec) followed by sorting for CD44^{lo}CD62L^{hi}V α 2⁺V β 5⁺*Thpok*-GFP⁺ CD4⁺ cells using a FACS Aria cell sorter. Naïve OT-II *Thpok* GFP⁺ CD4⁺ cells (1×10^6) were transferred into the *Rag1*^{-/-} recipients via retro-orbital route. To activate the antigen-specific OT-II T cells, these *Rag1*^{-/-} recipients were fed an ovalbumin-containing diet for 4 weeks.

ChIP and tiling array

Chip-on-chip assay was performed as previously described¹⁹. In brief, 10×10^6 CD4 SP thymocytes from FH-ThPOK transgenic mice were used to isolate chromatin DNA for chromatin immuno-precipitation with anti-Flag and amplified twice by ligation-mediated PCR according to the manufacturer's protocol (Agilent). Custom microarray generated by Agilent that tiled through the murine *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.

Chromatin immunoprecipitation (ChIP)

Cells were fixed with a 10% formaldehyde solution for 10 min, followed by cell lysis and sonication to solubilize and shear cross-linked chromatin DNA. The resulting whole cell extract was incubated with anti-mouse Ig or anti-rabbit Ig beads (Invitrogen) and the appropriate antibody, washed and bound complexes were eluted from the beads. Cells were fixed with a 10% formaldehyde solution for 10 min, followed by cell lysis and sonication to solubilize and shear cross-linked chromatin DNA. The resulting whole cell extract was incubated overnight at 4 °C with anti-mouse Ig or anti-rabbit Ig beads (Invitrogen) and the appropriate antibody. Beads were then washed 5 times with RIPA buffer and once with TBS. Bound complexes were eluted from the beads and cross-linking was reversed by overnight incubation at 65 °C. The primers used are as follows: E8_I-forward 5'-CAA TGC GAA TGT GAC TCA AG-3', E8_I-reverse 5'-TAA TGC GGT GTG ATC AGT ATG-3', *Thpok* silencer-forward 5'-TGG TTT CGA GAC TGG CTG GT-3' and *Thpok* silencer - reverse 5'-GAC CGA GGA GCT GCT TTC AG-3'.

Retroviral transduction of CD4 T cells

Transduction of *Thpok*, *Thpok*-HD or Cre retroviral vectors into primary CD4 T cells was performed as previously described⁴⁷. *Thpok* vectors were a gift from D. Kappes (Fox Chase Cancer Center, PA). The transfected cells were purified using a FACS Aria flow cytometer, and $1-2 \times 10^6$ cells were transferred into *Rag1*^{-/-} mice. Four to five weeks after transfer, tissues were recovered from recipient mice for flow cytometry analyses.

Primers for qPCR

GATA-3-forward 5'-AGG ATG TCC CTG CTC TCC TT-3', GATA-3-reverse 5'-GCC TGC GGA CTC TAC CAT AA-3', ThPOK-forward 5'-ATG GGA TTC CAA TCA GGT CA-3', ThPOK-Reverse 5'-TTC TTC CTA CAC CCT GTG CC-3', T-bet-forward 5'-ATC

CTG TAA TGG CTT GTG GG-3', T-bet-Reverse 5'-TCA ACC AGC ACC AGA CAG AG-3', CRTAM-forward 5'-TTA GAG TGA GCG TTT GGC CT-3', CRTAM-Reverse 5'-GGG AGT CCT CAG TTT GCT GT-3', ROR γ T-forward 5'-CCG CTG AGA GGG CTT CAC-3', ROR γ T-Reverse 5'-TGC AGG AGT AGG CCA CAT TAC A-3', L32 -forward 5'-GAA ACT GGC GGA AAC CCA-3', L32 -Reverse 5'-GGA TCT GGC CCT TGA ACC TT-3', IL23R-forward 5'-TTC AGA TGG GCA TGA ATG TTT CT-3', IL23R--Reverse 5'-CCA AAT CCG AGC TGT TGT TCT AT-3', IL17A-forward 5'-TGA GAG CTG CCC CTT CAC TT-3', IL17A-Reverse 5'-ACG CAG GTG CAG CCC A-3', IL-22-forward 5'-CAG GAG GTG GTA CCT TTC CTG A-3', IL-22-Reverse 5'- TCT GGT CGT CAC CGC TGA T-3', IL-2-forward 5'-CCT GAG CAG GAT GGA GAA TTA CA-3', IL-2-Reverse 5'-TCC AGA ACA TGC CGC AGA G-3', IL-4 -forward 5'-ACA GGA GAA GGG ACG CCA T-3', IL-4-Reverse 5'- GAA GCC CTA CAG ACG AGC TCA-3', IL-13-forward 5'-AGA CCA GAC TCC CCT GTG CA-3', IL-13-Reverse 5'-TGG GTC CTG TAG ATG GCA TTG-3', GRANZYME A-forward 5'-ATT CCT GAA GGA GGC TGT GAA-3', GRANZYME A-Reverse 5'-GCA GGA GTC CTT TCC ACC AC-3', GRANZYME B-forward 5'-GCC CAC AAC ATC AAA GAA CAG-3', GRANZYME B-Reverse 5'-AAC CAG CCA CAT AGC ACA CAT-3', CD8 α -forward 5'-ACT GCA AGG AAG CAA GTG GT-3', CD8 α -Reverse 5'-CAC CGC TAA AGG CAG TTC TC-3', IFN γ - forward 5'- ATG AAC GCT ACA CAC TGC ATC -3', IFN γ -reverse 5'- CCA TCC TTT TGC CAG TTC CTC -3'.

CD107 mobilization assay

The degranulation activity of cytolytic cells was measured as described⁴⁸. Briefly, enriched polyclonal IELs were activated with plate-bound anti-TCR β (2 μ g/ml) for 4 h in the presence of CD107 antibody and Brefeldin-A in 96-well U bottom plates at 37 °C and 5% CO₂. OT-II TCR transgenic IELs were co-cultured with LPS overnight-treated LB27.4 (Mouse B cell lymphoma) APCs in 5:1 ratio and MHC-II class II specific OVA₃₂₃₋₃₃₉ peptide or MHC-I specific SIINFEKL peptide for 6 h in the presence of CD107 antibody and Brefeldin-A in 96-well V bottom plates at 37 °C and 5% CO₂. In some cases, sorted IEL were cultured for 3 days in the presence of recombinant mouse IL2 (BD Biosciences, USA) and recombinant human IL-15 (R&D systems Inc.). Following stimulation, CD107 and cytokine expression was assessed by flow cytometry.

In vitro cytotoxicity assay

Cytotoxicity by anti-TCR β cross-linked T cells was measured 6 h after coculturing with P815 target cells at 37 °C and 5% CO₂ using the CytoTox 96 Non-Radioactive cytotoxicity assay kit (Promega). The % cytotoxicity was calculated based on the release of lactate dehydrogenase. In some cases, cultured IELs were used.

Statistical Analysis

Data are presented as mean \pm SEM. Comparisons between groups used the Student's *t* test assuming two-tailed distributions unless otherwise stated, with an alpha level of 0.01–0.05. In some samples Nonparametric two tailed Mann-Whitney test or ANOVA and Bonferroni as post-test were used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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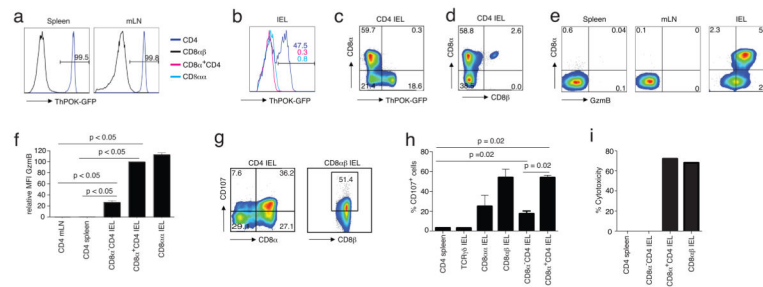


Figure 1.

Some mature CD4 T cells do not maintain ThPOK expression in the periphery. **(a)** Frequency of GFP positive cells among gated $CD45^{+}TCR\beta^{+}$ lymphocytes isolated from the spleen and mLN of naïve ThPOK-*gfp* reporter mice. **(b)** Frequency of GFP positive cells among gated $CD45^{+}TCR\beta^{+}$ lymphocytes isolated from the small intestine epithelium of naïve ThPOK-*gfp* reporter mice. Data in **a** and **b** are representative of 2 independent experiments, $n = 4$ or 5 mice. **(c)** Cell surface staining for CD8 α and GFP expression in gated $CD45^{+}TCR\beta^{+}CD8\beta^{-}CD4^{+}$ IELs as in **(b)**. **(d)** Cell surface staining for CD8 α and CD8 β expression in gated $CD45^{+}TCR\beta^{+}CD4^{+}$ IELs as in **(b)**. **(e)** Cell surface staining for CD8 α and intracellular for Granzyme B in gated $CD45^{+}TCR\beta^{+}CD8\beta^{-}CD4^{+}$ lymphocytes isolated from spleen, mLN and sIELs of naïve WT B6 mice. Values in plots indicate percent positivity **(f)** MFI for Granzyme B expression by $CD4^{+}$, $CD8\alpha^{+}CD4^{+}$ or $CD8\alpha\alpha^{+}$ T cells in gated $CD45^{+}TCR\beta^{+}CD8\beta^{-}$ lymphocytes from different tissues of naïve wild type B6 mice. Data in **c–f** are representative of 3 independent experiments, $n = 4$ or 5 mice. ANOVA and Bonferroni as post test were used for statistics **(g)** Frequency of CD107 positive cells among total gated $CD45^{+}TCR\beta^{+}CD8\beta^{-}CD4^{+}$ - and $CD45^{+}TCR\beta^{+}CD8\alpha\beta^{+}$ IELs after anti-TCR β stimulation. Representative data of 4 independent experiments. Values indicate percent positivity **(h)** Frequency of CD107 expressing $CD4^{+}$ splenocytes or $TCR\gamma\delta^{-}$, $CD8\alpha\alpha^{+}$, $CD8\alpha\beta^{+}$, $CD8\alpha^{-}$ and $CD8\alpha^{+}CD4^{+}$ IEL subsets after anti-TCR β stimulation. Data are representative of 4 independent experiments. Each bar shows standard error of the mean (SEM; $n=4$). Nonparametric two tailed Mann-Whitney test was used for statistics **(i)** % cytotoxicity of respective lymphocyte subsets as measured by LDH release assay. Representative data of 4 independent experiments.

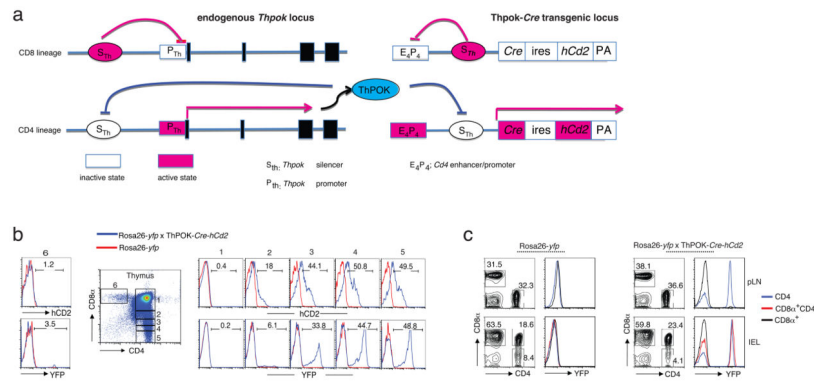


Figure 2. ThPOK⁻ mature CD4 T cells are progeny of ThPOK-expressing thymocytes. **(a)** A schematic presentation of the *Thpok* and the transgenic ThPOK-*Cre* locus in CD8- and CD4-lineage thymocytes. Red indicates “active” and white “inactive”. **(b)** Staining for CD4 and CD8 α (dot plot), human CD2 and YFP (histograms) of thymocytes gated on CD4 and CD8 α expression levels (gates 1–6) where 1 represents immature double-positive thymocytes and 5 and 6 represent mature, single-positive CD4 and CD8 thymocytes, respectively. Red histograms represent thymocytes from *Rosa26-yfp* mice and blue histograms from *Rosa26-yfp* x ThPOK-*Cre-hCd2* mice. Values above histograms indicate percent positivity **(c)** Staining for CD4 and CD8 α (contour plots) and YFP (histograms) of peripheral lymph node (pLN) and sIELs, gated on TCR β^+ CD4⁺ lymphocytes isolated from the *Rosa26-yfp* (left)- or the *Rosa26-yfp* x ThPOK-*Cre-hCd2* (right) mice. Values above histograms and in contour plots indicate percent positivity. Data in **b** and **c** are representative of at least 3 independent experiments.

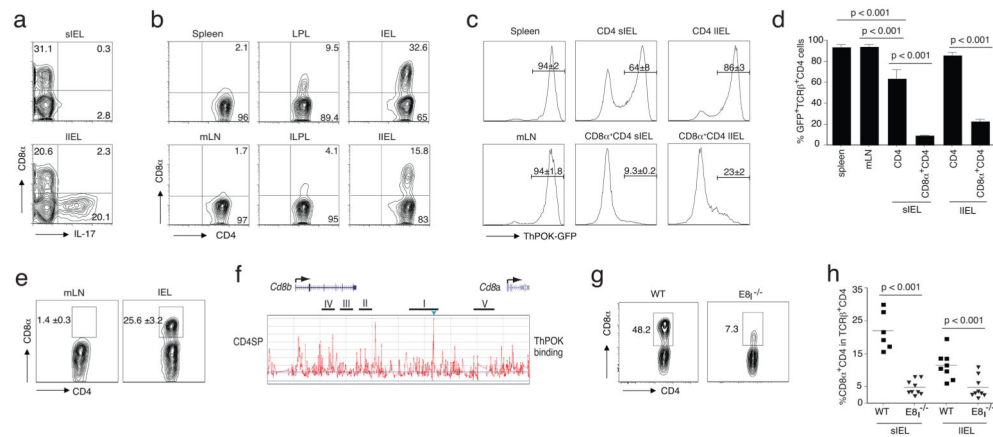
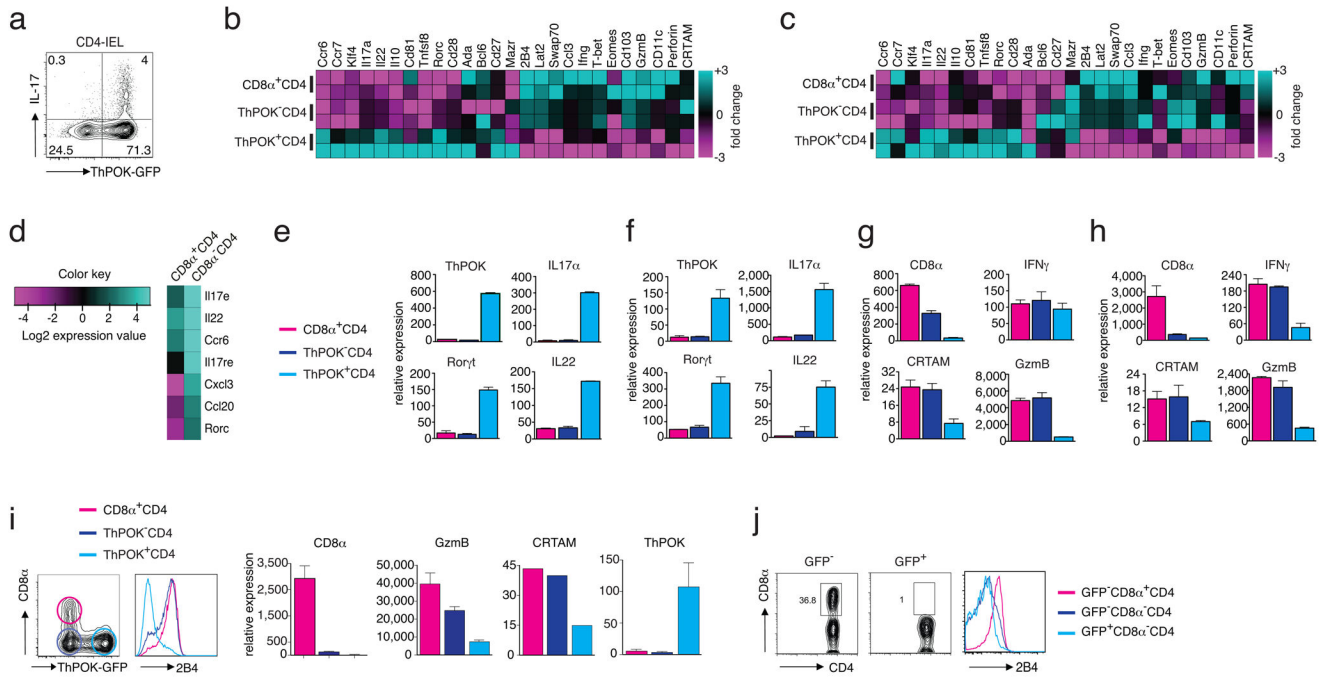


Figure 3.

ThPOK⁻ CD4 effector cells lose ThPOK as mature cells in the periphery. **(a)** CD8 α and intracellular IL-17 staining of gated CD45⁺TCR β ⁺CD4⁺ small and large intestine IELs of *Rag1*^{-/-} recipient mice 8 weeks after adoptive transfer of naïve TCR β ⁺CD8 α ⁻CD45RB^{high}CD25⁻CD4⁺ spleen T cells. Values in plots indicate percent positivity. **(b)** CD8 α and CD4 staining of gated CD45⁺TCR β ⁺CD4⁺ T cells from various tissues of *Rag1*^{-/-} recipient mice 8 weeks after adoptive transfer of naïve TCR β ⁺CD8 α ⁻CD45RB^{high}CD25⁻CD4⁺ spleen T cells. Values in plots indicate percent positivity. **(c)** *Thpok* GFP expression in gated CD45⁺TCR β ⁺ -SP CD4⁺ or -DP CD8 α ⁺CD8 β ⁻CD4⁺ lymphocytes isolated from various tissues of *Rag1*^{-/-} recipient mice 8 weeks after transfer of sorted TCR β ⁺CD8 α ⁻CD45RB^{high}CD25⁻CD4⁺ spleen T cells from *Thpok* GFP donor mice. Values above histograms indicate mean \pm SEM percent positivity. Data in **a–c** are representative of 3 independent experiments. **(d)** Frequency of GFP⁺ cells in gated CD45⁺TCR β ⁺ -SP CD4⁺ or -DP CD8 α ⁺CD8 β ⁻CD4⁺ lymphocytes from spleen, mLN or IELs of *Rag1*^{-/-} recipients after transfer of sorted naïve TCR β ⁺CD8 α ⁻GFP⁺CD45RB^{high}CD25⁻CD4⁺ spleen T cells from *Thpok* GFP donor mice. Data are representative of 3 independent experiments. ANOVA and Bonferroni as post test were used for statistics. Each bar shows the SEM **(e)** Surface Staining for CD8 α on re-transferred sorted (> 99.7% purity) CD45⁺TCR β ⁺CD8 α ⁻CD4⁺ donor IELs (from 3 pooled recipient mice) isolated from mLN and sIELs of a second *Rag1*^{-/-} recipient 8 weeks after the second transfer. Values in plots indicate mean \pm SEM percent positivity. Data are representative of 2 independent experiments. **(f)** Chromatin immuno-precipitation (ChIP) with tiling array using CD4 SP thymocytes from the FH-ThPOK knock-in mice expressing FLAG-hemagglutinin epitope tagged ThPOK protein. Binding of ThPOK to the E8₁ region was marked with a green arrowhead. Data are representative of 2 independent experiments. **(g)** Frequency of CD8 α positive CD45⁺TCR β ⁺CD8 β ⁻CD4⁺ IELs isolated from wild type (left panel) and E8₁-deficient (right panel) mice. Data are representative of 3 independent experiments, n = 4 or 5 mice. **(h)** Frequency of CD8 α ⁺CD4⁺ IELs from *Rag1*^{-/-} recipient mice 8 weeks after transfer of wild type or E8₁-deficient TCR β ⁺CD8 α ⁻CD45RB^{high}CD25⁻CD4⁺ spleen T cells.

**Figure 4.**

Activated CD4 T_H cells that lose ThPOK expression differentiate to CTL. **(a)** Intracellular IL-17 and GFP staining in gated CD45⁺TCR β ⁺CD4⁺ IELs from Rag1^{-/-} recipient mice of Rag1^{-/-} recipients that previously received sorted naïve TCR β ⁺CD8 α ⁻GFP⁺CD45RB^{high}CD25⁻CD4⁺ spleen T cells from ThPOK-*gfp* donor mice. Data are representative of 3 independent experiments. **(b)** Gene expression microarray analysis of mRNA from sorted CD45⁺TCR β ⁺-ThPOK⁺CD8 α ⁻CD4⁺, -ThPOK⁻CD8 α ⁻CD4⁺ and ThPOK⁻CD8 α ⁺CD4⁺ IELs of naïve ThPOK-*gfp* reporter mice. Each sample represents 2 independent experiments, sorting and duplicate microarray analyses **(c)** Gene expression microarray analysis of mRNA from sorted CD45⁺TCR β ⁺-ThPOK⁺CD8 α ⁻CD4⁺, -ThPOK⁻CD8 α ⁻CD4⁺ and ThPOK⁻CD8 α ⁺CD4⁺ IELs of Rag1^{-/-} recipients that previously received sorted naïve TCR β ⁺CD8 α ⁻ GFP⁺CD45RB^{high}CD25⁻CD4⁺ spleen T cells from ThPOK-*gfp* donor mice. Each sample represents 2 independent experiments, sorting and duplicate microarray analyses **(d)** Heat map of normalized gene expression levels of Th17 signature genes in donor CD4 T cells determined by microarray analysis as in **(b)**. **(e)** Relative mRNA levels of Th17-associated genes from sorted T cell subsets as in **(c)**. **(f)** Relative mRNA levels of Th17-associated genes from sorted T cell subsets as in **(d)**. **(g)** Relative mRNA levels of CTL-associated genes from sorted T cell subsets as in **(c)**. **(h)** Relative mRNA levels of CTL-associated genes from sorted T cell subsets as in **(d)**. **(i)** ThPOK-GFP (left panel) and 2B4 (right panel) expression in gated CD45⁺TCR β ⁺CD4⁺ IELs as in **(a)**. Relative mRNA expression of CD8 α , Granzyme B, CRTAM and ThPOK in IELs sorted for (CD45⁺TCR β ⁺CD4⁺) GFP⁺, GFP⁻CD8 α ⁻, and GFP⁻CD8 α ⁺ subsets isolated from 4 individual Rag1^{-/-} recipients as in **(a)**. Data are representative of 3 independent experiments. Gene expression was analyzed by quantitative real-time PCR and expressed relative to L32 as an internal standard. **(j)** Staining for CD4, CD8 α (left and middle panels) and 2B4 (right panel) of gated TCR β ⁺CD4⁺ IELs from Rag1^{-/-} recipients that previously

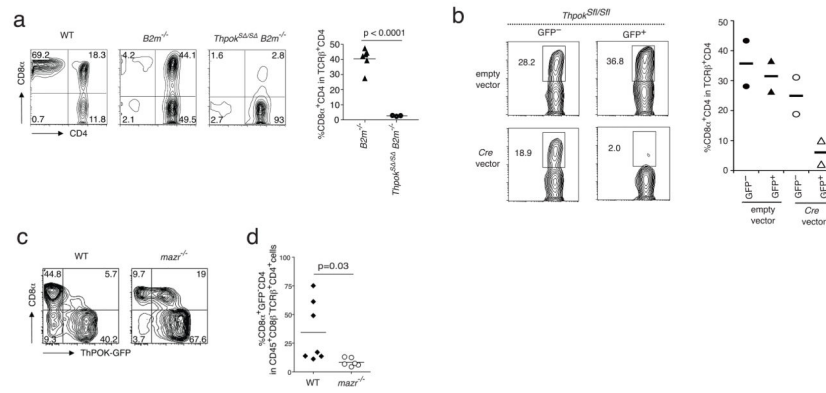
received ThPOK-transfected (GFP⁺) or untransfected (GFP⁻) TCR β ⁺CD8 α ⁻CD45RB^{high}CD25⁻CD4⁺ donor spleen T cells. Data are representative of 3 independent experiments, n = 3 mice.

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**Figure 5.**

The *Thpok* silencer forms the switch that terminates *Thpok* expression in mature CD4 T cells. **(a)** Staining for CD4 and CD8 α expression on gated TCR β^+ CD4 $^+$ sIELs isolated from naïve wild type, B2m $^{-/-}$ and *Thpok*^{S/S} (*Thpok* silencer deletion) x B2m $^{-/-}$ mice. Plots are representative of 3 independent experiments, n = 2 or 3 mice. **(b)** Staining as in **(a)** gated on (GFP $^+$ or GFP $^-$) sIELs isolated from *Rag1* $^{-/-}$ recipients 4–5 weeks after adoptive transfer of *Cre-gfp*-transfected TCR β^+ CD4 $^+$ *Thpok*^{Silox/Silox} (*Thpok*^{Silox/Silox}) primary cells. Data are representative of 2 independent experiments. **(c)** Staining for GFP and CD8 α expression on sIELs gated as in **(a)** and isolated from *Rag1* $^{-/-}$ recipients after transfer of sorted naïve TCR β^+ CD8 α^- GFP $^+$ CD45RB^{high}CD25 $^-$ CD4 $^+$ spleen T cells from wild type- and *Mazr* $^{-/-}$ ThPOK-*gfp* donor mice. Representative data of 3 independent experiments. **(d)** Frequency of CD8 α^+ GFP $^-$ CD4 sIELs as in **(c)**. Data are representative of 3 independent experiments.

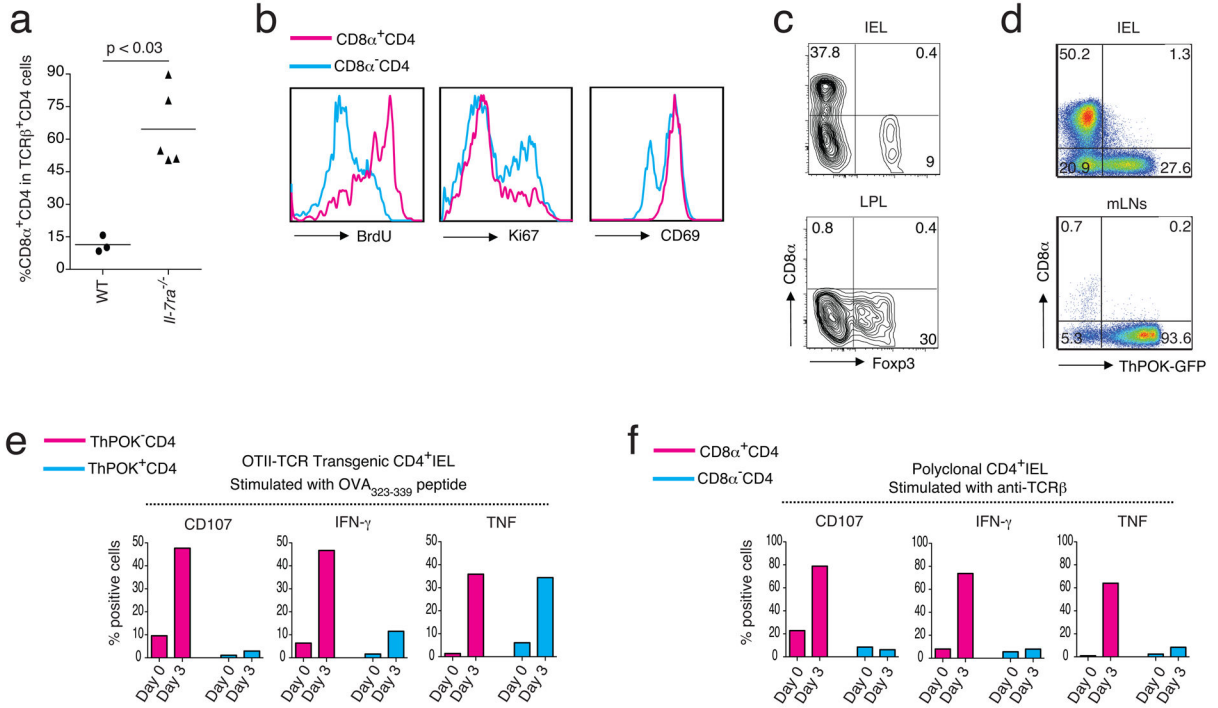


Figure 6. The ThPOK loss and reprogramming of CD4 CTL is an antigen-driven process *in vivo*. **(a)** Frequency of CD8 α ⁺CD4⁺ T cells in gated CD45⁺TCR β ⁺CD8 β ⁻CD4⁺ IELs from wild type or *Il-7ra*^{-/-} mice. Nonparametric two-tailed Mann-Whitney test was used for statistics. **(b)** BrdU staining following an i.p. injection with 1 mg BrdU and 6 days of 0.8 mg/ml BrdU in the drinking water (left panel). Ki67 staining (middle panel) and CD69 staining (right panel) of CD8 α ⁺ or CD8 α ⁻ gated CD45⁺TCR β ⁺CD8 β ⁻CD4⁺ IELs of naïve wild type mice. **(c)** Staining for CD8 α and Foxp3 in gated CD45⁺TCR β ⁺CD8 β ⁻CD4 IELs (upper panel) and LPL (lower panel) of OVA-specific OTII TCR transgenic *Rag1*^{-/-} mice after 4 weeks of OVA-diet feeding. Representative data of 2 independent experiments, n = 4 mice per group. **(d)** Frequency of CD8 α and GFP positive cells among CD45⁺TCR β ⁺ OT-II TCR ThPOK-*gfp* CD4⁺ IELs isolated from *Rag1*^{-/-} recipient mice after 4 weeks of OVA-diet feeding. Data represents 5 independent experiments. **(e)** Frequency of CD107, IFN γ and TNF α positive cells in ThPOK⁻ (red) and ThPOK⁺ (blue) CD45⁺TCR β ⁺ OT-II TCR ThPOK-*gfp* CD4⁺ IELs as in **(d)** analyzed at day 0, without IL-15 exposure (red bars) or after 3 days *in vitro* culturing with rIL-15 (blue bars) prior to re-stimulation with OVA₃₂₃₋₃₃₉ peptide. Data are representative of 3 independent experiments. **(f)** Frequency of CD107, IFN γ and TNF positive cells in CD8 α ⁺ (red) and CD8 α ⁻ (green) CD45⁺TCR β ⁺CD8 β ⁻CD4⁺ polyclonal wild type IELs without or with IL-15 as in **(e)**. Data are representative of 3 independent experiments.