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CD4 CTL: Living up to the challenge

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Abstract

During thymic development, thymocytes expressing a T cell receptor consisting of an alpha and beta chain (TCR $\alpha\beta$), commit to either the cytotoxic- or T helper-lineage fate. This lineage dichotomy is controlled by key transcription factors, including the T helper (Th) lineage master regulator, the Th-inducing BTB/POZ domain-containing Kruppel-like zinc-finger transcription factor, ThPOK, (formally cKrox or Zfp67; encoded by *Zbtb7b*), which suppresses the cytolytic program in major histocompatibility complex (MHC) class II-restricted CD4⁺ thymocytes and the Runt related transcription factor 3 (Runx3), which counteracts ThPOK in MHC class I restricted precursor cells and promotes the lineage commitment of CD8 $\alpha\beta$ ⁺ cytolytic T lymphocytes (CTL). ThPOK continues to repress the CTL gene program in mature CD4⁺ T cells, even as they differentiate into effector Th cell subsets. The Th cell fate however is not fixed and two recent studies showed that mature, antigen-stimulated CD4⁺ T cells have the flexibility to terminate the expression of ThPOK and functionally reprogram to cytotoxic effector cells. This unexpected plasticity of CD4 T cells results in the post-thymic termination of the Th lineage fate and the functional differentiation of distinct MHC class II-restricted CD4⁺ CTL. The recognition of CD4 CTL as a defined separate subset of effector cells and the identification of the mechanisms and factors that drive their reprogramming finally create new opportunities to explore the physiological relevance of these effector cells *in vivo* and to determine their pivotal roles in both, protective immunity as well as in immune-related pathology.

Keywords

CD4 T helper cells; CD8 $\alpha\beta$ Cytotoxic T lymphocyte; ThPOK; Runx3; plasticity; lineage commitment; functional reprogramming; tumor; chronic viral infection; intestine; autoimmunity; protective immunity

1. Introduction

MHC class II restricted CD4⁺ TCR $\alpha\beta$ ⁺ cells have commonly been classified as ‘helper’ T cells based on their function to help promote or dampen cellular or humoral immune responses whereas MHC class I restricted CD8 $\alpha\beta$ ⁺ T lymphocytes are known to provide direct protection by killing infected or transformed cells. Nevertheless, numerous observations suggest that CD4 T cells may also play direct and indispensable roles in protective immunity. The functional dichotomy of TCR $\alpha\beta$ T cells is initially induced during

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thymic development and coincides with, but does not depend on, the specific MHC restriction and CD4- or CD8 $\alpha\beta$ co-receptor expression. The molecular mechanism, which controls the lineage-commitment is driven by the action and counteraction of key transcription factors such as the Th cell transcription factor, ThPOK, which drives the CD4⁺ Th cell fate and prevents thymocytes from differentiating into CTL [1–7]. Runx3, a member of the Runx family of transcription factors, has the opposite effect and terminates CD4 expression while promoting differentiation of MHC class I restricted thymocytes into CD8 $\alpha\beta$ CTL [8, 9]. ThPOK, continues to be expressed in naïve CD4 T cells and cooperates with additional transcription factors, such as T-bet, Gata3, ROR γ t or Foxp3, as activated mature CD4 T cells differentiate into Th1, Th2, Th17 or T regulatory cells respectively [4, 7].

Nevertheless, cytotoxicity associated with antigen-experienced CD4⁺ lymphocytes has been described for decades especially under conditions of chronic viral infections or viral-induced immune pathology and autoimmunity [10, 11]. At first cytolytic CD4⁺ T cells were considered a potential artifact of *in-vitro* generated T cell lines and clones but more recent reports provided unambiguous evidence that *in-vivo* some antigen-specific CD4⁺ T cells possess direct MHC class II restricted cytotoxic activity [12–18]. These cells were merely viewed however as functional variants of the classical CD4 Th1 cells and consequently they remained unexplored and their physiological relevance was often doubted. Because of this, cytolytic CD4 effector cells have not been fully appreciated nor recognized as possible active contributors in health and disease.

Recently two studies [4, 19], determined that cytotoxic CD4⁺ T cells form a separate type of CD4 effector cells that is distinct from any known conventional CD4⁺ Th subset. They showed that, like classical CD8 $\alpha\beta$ CTL, these mature CD4⁺ T cells lack expression of the Th master regulator, ThPOK. However, in contrast to the thymic committed CD8 $\alpha\beta$ CTL, termination of the *Thpok* gene expression in the cytotoxic CD4⁺ T cells occurs post-thymically in response to repeated stimulation with their cognate antigen *in-vivo* [4]. As a consequence of the loss of ThPOK expression, activated CD4⁺ T cells de-repress the cytolytic-gene expression program leading to the functionally effector differentiation of MHC class II restricted CD4 CTL. The post-thymic reprogramming of mature CD4⁺ T cells provides a unique mechanism of plasticity not only to generate cytotoxic MHC class II restricted effector T cells but also to redirect Th cells away from becoming either inflammatory- or immunosuppressive cells.

The discovery of the CTL reprogramming of mature CD4⁺ T cells not only represents a major advance in our understanding of T cell biology but also provides powerful opportunities for the design of new strategies to overcome inflammatory T cell-mediated pathologies or immune suppression as well as to induce pre-existing anti-viral or anti-tumor protective immunity. Together with the observation that defects in the differentiation or regulation of this process may lead to impaired immune protection or aberrant immune functions [19], these significant novel insights have evoked new interest in the cytotoxic CD4⁺ T cells as potential important beneficial and/or pathogenic contributors of the immune response.

2. Thymic commitment and lineage decision

The thymus is initially seeded by bone marrow-derived uncommitted progenitors, which gradually lose their multipotency and fully commit to the T cell lineage. The initial process involves suppression of gene expression programs characteristic of other lineages as well as the induction of a T cell specific gene expression profile mediated by various transcription factors, including Runx1, Gata3 and E-box proteins, which cooperate with Notch1 to initiate

T-lineage differentiation [20]. Immature thymocytes first appear as CD4 and CD8 $\alpha\beta$ coreceptor double negative (DN) cells that sequentially transition through various stages defined by the expression of CD44 and CD25 as CD44⁺CD25⁻ DN1, CD44⁺CD25⁺ DN2, CD44⁻CD25⁺ DN3 and finally CD44⁻CD25⁻ DN4 cells. During the immature DN stages the T lineage committed progenitors rearrange the loci encoding the TCR and pass through various checkpoints to verify proper rearrangement of the γ and δ or α and β TCR chains. Before expressing a full $\alpha\beta$ TCR, precursor thymocytes first express a pre-TCR consisting of the CD3 components together with a variable rearranged β -chain and an invariant pre- α chain. Signals received through the pre-TCR, known as β -selection, direct $\alpha\beta$ TCR progenitors to the next phase and those that pass β -selection undergo a proliferative burst and become CD4 CD8 $\alpha\beta$ double positive (DP) or triple positive (TP) thymocytes that express CD8 $\alpha\alpha$ together with the coreceptors CD8 $\alpha\beta$ and CD4 [21]. During this phase, the T cell progenitors also rearrange the *Tcra* gene, resulting in the surface expression of mature $\alpha\beta$ TCR complexes and the full commitment of the thymocyte to the TCR $\alpha\beta$ lineage. Those thymocytes, which successfully rearrange an $\alpha\beta$ TCR progress further through a selection process based on the interaction of their TCR with self-MHC and self-antigens. Selected thymocytes segregate into mature DN or 'single-positive' (SP) cells that either express the CD8 $\alpha\beta$ coreceptor together with an MHC class I restricted TCR or the CD4 coreceptor in conjunction with an MHC class II specific TCR. The selection event also coincides with, but does not depend on, the functional commitment of the developing thymocytes, to either the CD8 $\alpha\beta$ CTL-lineage or the CD4 Th-lineage. The functional fate decision is mediated by key transcription factors, including the master Th transcription factor, ThPOK, which promotes the CD4⁺ T helper fate and prevents thymocytes from differentiating into CTL and *Runx3*, which has the opposite effect and terminates CD4 expression while driving differentiation of the maturing thymocyte into the CD8 $\alpha\beta$ CTL lineage [20] (Fig. 1).

3. Lineage-fate decisions and transcription factors

Although much attention has been focused on ThPOK as the master transcription factor of the CD4 Th lineage fate, another important regulator of early T cell development, β -selection and CD4 Th lineage differentiation is the zinc finger transcription factor GATA-binding protein 3 (*Gata3*) [3, 5, 22]. *Gata3* is expressed throughout T cell development and asymmetrically distributed during thymic selection with increased levels in CD4 Th-lineage differentiating thymocytes [23], but reduced levels in CD8 $\alpha\beta$ CTL committed progenitors [3, 24]. The increase in *Gata3* expression also occurs in *Thpok*-deficient, MHC-II-restricted precursor cells whereas in contrast, ThPOK is not detected in *Gata3*-deficient thymocytes, indicating that *Gata3* functions upstream of ThPOK as a critical inducer of *Thpok* in early T cell development. Since, enforced *Thpok* transgene expression in *Gata3*-deficient thymocytes does not lead to CD4-Th lineage commitment [25], it also suggests that *Gata3* serves additional roles to promote the ThPOK-driven CD4 Th fate. Importantly, *Gata3* also represses CD8-lineage gene- expression, including *Runx3*. Consequently, high-level of *Gata3* and *Runx3* are mutually exclusive, making *Gata3* a critical regulator of the CD4-CD8 lineage commitment. Interestingly, even though *Gata3* cooperates with ThPOK to promote the CD4 Th-fate, *Gata3* is not required for the ThPOK-mediated suppression of *Runx3*, and transgenic expression of ThPOK alone is sufficient to block the CD8 differentiation of *Gata3*-deficient thymocytes [25]. Furthermore, enforced *Thpok* expression causes precursor cells, including MHC class I-restricted thymocytes, to develop into CD4 Th cells [6, 25]. Similarly, in mice expressing ThPOK constitutively due to *Runx* deficiency, class I-restricted CD4-expressing lymphocytes develop, which exhibit phenotypic and functional characteristics of CD4⁺ Th cells [26, 27]. Conversely, in mutant mice, which fail to express functional ThPOK (ThPOK^{HD/HD}, a mutant form of *Thpok*, found in CD4 Th-deficient mice), MHC class II-restricted CD8 T cells develop, which exhibit a functional CTL phenotype [2], whereas the *Cd4* silencer in these cells is derepressed [28]. These findings

demonstrate that ThPOK is necessary and sufficient to suppress the CD8 $\alpha\beta$ lineage fate in developing thymocytes. The re-expression of CD8 in redirected CD4 Th- cells is driven by Runx3; which is an essential transcription factor to drive expression of CD8 $\alpha\beta$ CTL-lineage associated genes like *perforin* and *granzyme b* the cytokine *ifn- γ* and the transcription factor *eomesodermin (eomes)* [7, 29]. Runx3 protein is not detected in DP thymocytes, but it is up-regulated during the differentiation of CD8 CTL-lineage cells in the thymus and remains expressed in post-thymic mature CD8 $\alpha\beta^+$ T cells [9, 30, 31] (Fig. 1); Although *Runx3* deletion does not prevent CD8 CTL differentiation, it results in reduced numbers of CD8 $\alpha\beta^+$ T cells, and most of them appear as CD4CD8 $\alpha\beta$ DP cells. Furthermore, Runx3-deficient CD8 cells have reduced CD8 expression [30], consistent with the direct binding of Runx3 to the Cd8 enhancer, E8_I, located in the *Cd8a* gene control region [31]. The presence of CD4 Th lineage cells in *Thpok-Runx* double-deficient mice, but not in *ThPOK* single-deficient mice, suggests that Runx not only directs the CTL lineage but also inhibits the expression of some Th genes that direct *Runx*-deficient thymocytes toward the CD4 Th lineage. In line with this, Runx3 is able to bind a silencer located upstream of the distal promoter of the *Thpok* gene, which results in active repression of ThPOK in immature CD8 $\alpha\beta^+$ thymocytes [9, 30–32]. This negative feed back loop system, where ThPOK represses Runx3 during the Th lineage commitment and Runx represses ThPOK in CTL-lineage thymocytes, prevents coexpression of both transcription factors in committed thymocytes (Fig. 1 and 2). Runx3 does not require MHC-I-specific signals for its initial induction in thymocytes since it is expressed in MHC-II restricted thymocytes that are *Thpok*-deficient [33]. On the other hand, *Thpok* is kept silent in MHC-I-restricted thymocytes before detectable Runx3 up-regulation, whereas enforced expression of Runx3 does not redirect MHC-II-restricted thymocytes into the CD8 $^+$ CTL-lineage [34, 35]. These observations indicate that the mutual exclusive regulation of ThPOK and Runx3 is not the only mechanism that drives the lineage choice in developing thymocytes and that additional factors are involved to direct the reciprocal commitment of CD4 $^+$ and CD8 $\alpha\beta^+$ progenitor cells [26]. Such factors may include Mazr, a BTB/POZ domain-containing transcription factor, which also controls thymocyte fate [36] by repressing ThPOK expression in MHC class-I signaled DP thymocytes or NF- κ B, and E-box binding proteins, as well as genes involved in the Notch pathway, all of which have been proposed to be involved, positively or negatively, in the CD4/CD8 lineage fate [37–41].

4. Transcriptional regulation of mature CD4 $^+$ Th cells in the periphery

Although the mutual exclusive expression of the lineage master transcription factors is strictly regulated in the thymus, the control of these and other factors to maintain the lineage integrity—and prevent derepression of alternative gene programs in mature T cells is more flexible. For example, in activated CD4 $^+$ T cells, Runx3, which counteracts ThPOK in the thymus, is induced by T-bet and actively promotes the IFN- γ production by Th1 cells [42, 43]. On the other hand, conditional disruption of ThPOK expression post thymically in CD4 $^+$ T cells causes CD8 re-expression and the induction of granzyme B [7], whereas enforced expression of ThPOK in mature T cells results in lineage conversion of MHC class I-restricted T lymphocytes toward the CD4 fate [6]. These observations indicate that ThPOK expression, initially established in the thymus, must be maintained in the periphery in order to prevent the activation-induced Runx3-driven CTL program in CD4 $^+$ effector cells [7, 44]. In response to antigens, CD4 $^+$ T cells differentiate to various effector subtypes, controlled by master transcription regulators that direct the functional specialization of each specific Th subtype, including T-bet for Th1, Gata3 for Th2 cells and ROR γ t for Th17 (Fig.3). In general, primary transcription factors will induce a secondary set, such as Gata3-induced ThPOK or T-bet-induced Runx3, after which they cooperate to drive specific gene expression profiles like the Gata-3/ThPOK-driven Th program in thymocytes or the T-bet/Runx3 Th1 program in mature CD4 $^+$ T cells. Gata3 is no longer expressed in naïve or

resting mature CD4 T cells, however it is auto-induced upon activation [45], suggesting that Gata3 might have additional roles for the functional differentiation of CD4 effector cells. Consistent with this notion, conditional deletion of *Gata3* abolishes Th2 differentiation [46] and leads to the instability of Foxp3 Tregs during inflammation [47, 48]. In contrast, enforced Gata3 induced co-expression of Th2 cytokines in Th17 conditioned cells [49] and under Th1 conditions, Gata3 is redistributed to T-bet-binding sites at Th1 genes away from Th2 genes [50]. Hence, in activated CD4⁺ T cells, Gata3 functions as an inducer or repressor of functional gene expression programs in various CD4 Th subsets. However, even though Gata3 initially induces ThPOK expression in developing thymocytes, it is no longer required for the constitutive expression and maintenance of ThPOK in naïve CD4 T cells [25]. Instead ThPOK regulates its own expression by repressing a silencer in its promoter thus counteracting Runx3-driven transcriptional suppression, which results in continuous expression of ThPOK in mature CD4 Th-lineage T cells (Fig. 2). Activated CD4 T cells however, lacking *Thpok*, in the presence of normal Runx3 activity still undergo multiple Th effector differentiation, except Th2 polarization, which is inhibited by the re-expression of Runx3. This observation indicates that Runx3 repression by ThPOK is not required for the Th differentiation per se; but rather, it is necessary to suppress Runx3 and preserve specific CD4 Th effector fates. Moreover, a number of studies showed that ThPOK not only counteracts Runx3 but also negatively regulates the expression of other CD8 CTL-lineage genes including *Cd8a*, *perforin*, *granzyme B* [44]. In addition, *eomes*, which promotes IFN- γ expression and cytotoxic effector differentiation of mature CD8 $\alpha\beta$ cells, is directly repressed by ThPOK [51–53]. These observations indicate that ThPOK preserves the Th fate by negatively regulating the CD8 $\alpha\beta$ CTL transcription program in activated mature CD4 T cells. Another transcription factor LRF is also required for the maintenance of the Th program in mature CD4 T cells, however in contrast to the *Thpok* deletion, disruption of both factors, ThPOK and LRF, resulted in a complete block of Th gene expression and Th cell polarization of MHC class II restricted mature T cells *in-vivo*. These data identified the ThPOK-LRF pair as master regulators of Th differentiation and function [54].

5. Diverting from the T helper path: CD4 CTL

Although, it is evident that ThPOK functions to continuously suppress the CTL program in mature CD4 Th lymphocytes, many reports have repeatedly indicated the presence *in-vivo* of CD4⁺ T cells with cytolytic features [55–57]. For example, at steady state, effector cells within the epithelium of the intestine show an enrichment for cytotoxic T cells, including CD4⁺ T cells [4, 19] and small numbers of cytolytic CD4⁺ T cells can be detected in the blood of healthy individuals [57]. In immune challenges, including viral infections [58], antitumor responses [59, 60] and autoimmune disorders [61, 62], cytotoxic CD4⁺ T cells with a functional phenotype typical of end-stage differentiated effector cells, significantly expand in the blood and peripheral tissues [57, 63]. The presence of CD4⁺ cytotoxic T cells in various species, including humans and rodents and their expansion in certain disease conditions, suggest that they might participate in protective and/or pathogenic immune functions. Nonetheless, direct evidence for unique non-redundant roles for these cells in any aspect of the adaptive immune response has not been generated, mainly because cytolytic CD4⁺ effector cells have usually been viewed and treated as functional variants of the conventional CD4 Th1 subtype. As a result, CD4⁺ CTL have not been actively targeted as part of clinical strategies to fight or prevent infections or other immune related diseases. New advances, however, indicating that CD4 T cells with cytolytic capacity are separate from any CD4 Th subset [4, 19], together with the fact that the efficiency of MHC-class II effector cells to kill target cells *in-vitro* matches that of classical CD8 $\alpha\beta$ CTL, underscores the notion that direct protective immunity might not only be the valuable characteristic of CD8 $\alpha\beta$ MHC class I, but also of CD4 MHC class II restricted TCR $\alpha\beta$ ⁺ T cells.

6. Functional reprogramming of mature CD4 Th cells to CTL

The functional features expressed by cytolytic CD4 T cells suggest that ThPOK does not suppress the Runx3-driven CTL gene expression program in those cells. In agreement with this, Mucida et al. (2013) and Reis et al. (2013), provided the first evidence that activated CD4⁺ T cells have the capacity to lose ThPOK and gain the Runx3-controlled CTL gene-expression program. Using an adoptive transfer approach these new reports showed that naïve CD4⁺ T cells isolated from *Thpok*-GFP reporter mice transferred into lymphopenic (*Rag1*^{-/-}) recipient mice, lost GFP expression indicating that these CD4 donor cells no longer expressed ThPOK. In parallel with the loss of ThPOK, the transferred cells acquired various CTL features, including the expression of granzymes, perforin, and IFN- γ , together with the cell surface induction of several receptors typical of natural killer cells and conventional CD8 $\alpha\beta$ CTL. This included 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 $\alpha\beta$ effector cells [64–66]. In contrast, in this transfer model of induced colitis, genes characteristic of Th17 cells, including those encoding the cytokines IL-17A, IL-17F and IL-22, the cytokine receptor IL-23R and the transcription factor ROR γ t, were expressed by many of the GFP⁺ThPOK⁺ pathogenic CD4 donor cells but not by the GFP⁻ subset. Moreover, analysis of genes characteristic of other CD4 Th types, such as Th1 and Th2 cells, demonstrated that the gene-expression profile of those ThPOK⁻ CD4 donor cells was different than that expressed by typical ThPOK⁺ CD4 Th effector subsets. The reciprocal expression of either ThPOK or the CTL signature genes in the transferred *Thpok*-GFP CD4 donor cells indicated that the generation of CD4 CTL coincides with post-thymic loss of ThPOK and also confirmed the notion that ThPOK continuously suppressed the CTL program in conventional Th cell. Furthermore, enforced expression via retroviral transduction of either wild type or a mutant form of *Thpok* (ThPOK^{HD/HD}) showed that this post thymic loss of ThPOK resulted in the derepression of the CTL program in mature CD4 effector cells. Using a genomic deletion approach, Mucida et al. (2013), identified the *Thpok* silencer as the critical switch that turned off *Thpok* transcription in mature CD4⁺ T cells. This was also confirmed in an adoptive transfer experiment using CD4 donor cells, in which a *loxP*-flanked *Thpok* silencer (*Thpok*^{Sfl/Sfl}) was deleted at the mature stage. CD4 donor cells with a *Thpok* silencer-deletion failed to differentiate to CTL upon transfer, which further emphasized the critical role of the *Thpok* silencer in terminating the transcription of *Thpok* as part of the CTL-differentiation process. In contrast to ThPOK, which suppresses its own silencer, the transcription factor MAZR activates the *Thpok* silencer [67], resulting in negative regulation of *Thpok* transcription (Fig. 2). Accordingly, MAZR-deficient CD4⁺ donor cells transferred into *Rag1*^{-/-} recipient mice showed impaired capacity to reprogram to CTL, indicating that transcriptional termination of *Thpok* expression was critical for the functional differentiation of MHC class II-restricted CD4⁺ CTL.

Nevertheless, although the loss of ThPOK expression is a key feature in diverting mature CD4 T cells to CTL, it is not sufficient and Reis et al. [19] showed using transfer of CD4 donor cells isolated from *Thpok*-GFP and *Runx3*-YFP double reporter mice, that both, the loss of ThPOK and the gain of Runx3 expression, were required for the reprogramming of CD4⁺ T cells to CTL. Interestingly, whereas in this transfer model of induced colitis, forced loss of ThPOK expression resulted in dampening of the inflammation, although it did not impair the Th17 differentiation, the lack of Runx3 expression due to a *Runx3* deletion resulted in severe pathology with enhanced Th17 differentiation. These observations indicated that the Runx3-driven differentiation of mature CD4⁺ Th cells to CTL diverts them away from becoming pathogenic effector cells. Some CD4⁺ T cells were Runx3^{hi}ThPOK^{hi}, however, none were Runx3^{lo}ThPOK^{lo}, suggesting that the induction of Runx3 occurred before the full downregulation of ThPOK. Furthermore, conditional deletion of Runx3 in T

cells indicated that the activation-induced Runx3 expression was critical for the generation of the ThPOK-negative CD4 CTL *in-vivo*. The gain of Runx3 expression also coincided with the appearance of the transcription factor T-bet, which is known to promote Runx3 expression in Th1 conditioned CD4 Th cells [19]. Nevertheless, CTL programming is not exclusively driven by T-bet-induced Runx3 expression and other CTL transcription factors, such as Eomes, may also be involved in the development of cytotoxic CD4⁺ T cells *in vivo*. In line with this, since ThPOK directly suppresses *eomes* in CD4⁺ Th cells, it is possible that in CTL precursor cells, which lose ThPOK, Eomes becomes expressed and operates either alone or in synergy with Runx3 to drive the functional reprogramming of fully functional CD4 CTL.

7. The CD4 CTL differentiation coincides with chronic activation

Th1-polarized, CD4⁺ effector cells can acquire perforin-mediated CTL activity *in-vitro* [68] and cytolytic CD4⁺ cells have been isolated *ex-vivo*, in response to acute influenza virus infections [11], however, the typical appearance of CD4 CTL *in-vivo* associates most often with chronic activation such as in chronic viral infections, including cytomegalovirus (CMV) [55], Epstein–Barr virus (EBV) [56] or Human Immune deficiency Virus (HIV) infections [57] or in autoimmune conditions such as rheumatoid arthritis [62]. This suggests that persistent antigen exposure may drive the progressive differentiation of CD4 CTL during which they lose the expression of *Thpok* and the typical Th program and derepress the genes encoding the functional cytotoxic phenotype. In line with this, Mucida et al. [4] showed that in mice, transgenic for a TCR (OT-II) specific for ovalbumin amino acids 323–339 [OVA (323–339)] presented by I-A^b or in *Rag1*^{-/-} recipients that previously received naïve OT-II CD4 donor T cells, that continuous feeding of an OVA-containing diet, resulted in the accumulation of a large number of OT-II CD4 cells in the intestine of these mice that expressed typical features of CTL. Furthermore, many OT-II *Thpok*-GFP cells isolated from reporter mice and transferred into OVA-fed *Rag1*^{-/-} recipient mice lost GFP expression indicating that they no longer expressed ThPOK. Instead they showed expression of typical cytolytic markers, such as 2B4 and granzyme B and they displayed an OVA-specific cytolytic response when restimulated *in-vitro* with the OT-II TCR-specific peptide OVA (323–339) but not when restimulated with the MHC class I-restricted OVA peptide SIINFEKL (amino acids 257–264) [4]. Interestingly, despite their cytolytic potential, OT-II ThPOK CD4⁺ effector cells remained immunologically quiescent *in-vivo* even in the continuous presence of OVA in the diet. However, similar to classical CD8αβ CTLs, which upon recall with their cognate antigen in the context of excess amounts of IL-15 as in active celiac disease [69, 70] differentiate into destructive killer cells [71] that produced large amounts of the inflammatory cytokines IFN-γ and tumor-necrosis factor (TNF) [69, 72, 73], chronic OVA diet-induced OT-II CD4 CTL restimulated *in-vitro* with their cognate antigen (Ag) and IL-15, also considerably upregulated their protective capacity, including a substantial increase of their cytolytic and cytokine response. Similar results were obtained using polyclonal CD4⁺ T cells isolated from normal lymphocyte-sufficient mice, which showed that natural *in-vivo* generated CD4 CTL also had the potential to substantially increase their cytolytic and inflammatory immune responses when restimulated with their cognate Ag in the presence of an inflammatory challenge. The ability to generate diet-Ag-induced CD4 CTL that accumulate within the intestinal epithelium and have the potential to display potent protective immune functions when challenged with their cognate diet Ag in the context of pathogen-induced danger signals, provides the mucosal immune system with a powerful arm to prevent invasion of newly encounter pathogens without pre-existing pathogen-specific immunity. Repeated Ag stimulation via the diet is likely not the only condition *in-vivo* that drives the reprogramming of CD4 Th cells to CTL, since cytotoxic CD4 T cells have frequently been observed in chronic viral infections, anti-tumor responses as well as in various autoimmune conditions. Therefore, it is possible that the repeated

stimulation with viral-, tumor- or self-antigens in the absence of excess inflammatory cytokines also leads to the loss of ThPOK and the functional differentiation of CD4 to CTL.

Overall, these new insights demonstrate that thymic committed CD4 Th cells have the flexibility, when chronically stimulated by their cognate Ag in the absence of inflammatory signals, to gradually differentiate into MHC class II restricted CTL that, although at steady state remain quiescent, have the potential to exert potent effector functions when reactivated with their Ag in the presence of inflammatory conditions.

8. CD4 CTL in health and disease

The functional differentiation of CD4 Th cells to CTL suggests that they have the capacity to participate in direct protective immunity to control infections or eliminate transformed cells. Especially the ability of cytolytic CD4 effector cells to function in an MHC class II restricted fashion, renders them potentially capable of detaining viral infections trophic for class II⁺ target cells. Furthermore, the gradual appearance of cytolytic CD4 effector cells in close association with persistent viral infections, when the CD8 response is weakened, inadequate or totally absent, suggests that CD4 CTL might be key immune cells for the control of chronic infections or acute secondary infections. Many viruses establish persistent infections, which are mostly innocuous [74]. However, in immune compromised individuals, they often reactivate and cause lethal diseases, such as the reactivation of CMV, a leading cause of mortality in AIDS patients and transplant recipients [75]. Although it is known that the cause of viral reactivation is due to suppression and/or deletion of protective immune cells, the specific type(s) of cell(s), which keep these persistent viruses under control in immune competent conditions, has not been identified. Nevertheless, even though the precise function of CD4 CTL in the maintenance of immunity against pathogens still remains to be established, the knowledge we have gained so far from *in-vitro* and *in-vivo* studies suggests strongly that they play non-redundant roles in controlling chronic viral infections. Consistent with this, studies with lymphocytic choriomeningitis virus (LCMV) in mice, which have led to profound insights that directly apply to viral infections in humans [76], have shown that in a chronic infection with LCMV clone 13, the time preceding viral clearance is associated with exhaustion of the CD8 CTL and an expansion of CD4⁺ T cells that are indispensable for controlling the chronic infection [77]. Similarly, CD4⁺ T cells also play a principal role in controlling persistent MCMV infections in the salivary gland in mice [78], and depletion of CD4, but not CD8 T cells, in immune competent animals significantly increased the accumulation of persistent MCMV [79]. Early in infection, MCMV-specific CD4 T cells display a typical Th1 phenotype (IFN γ ⁺/IL2⁺), whereas during latency, MCMV-specific cytolytic CD4 T cells (IFN γ ⁺/IL2⁻) emerge suggesting that CD4 CTL might be key to control persistent CMV infections [80, 81]. In agreement with this for example, the predominant specificity of human cytolytic CD4 T cells is for the CMV structural proteins, gB and gH [82, 83], which can be processed by endosomal proteases and loaded onto MHC class II molecules [84]. Furthermore, CD4⁺CD28⁻T cells isolated from the blood of individuals infected with CMV display direct antigen-specific cytotoxicity [63] and when analyzed *ex-vivo*, such cells were able to kill CMV-peptide pulsed autologous target cells in an MHC class II-restricted and pathogen-dependent manner without the need for prior *in-vitro* stimulation [55, 81, 84]. Similarly, CD4⁺ T cell clones derived from patients infected with EBV, were also shown to directly lyse EBV infected B cells, or transformed B cells expressing EBV proteins [56]. Furthermore, human cytolytic CD4⁺ T cell clones with specificity for CMV, EBV, Polyomavirus (BKV), vaccinia virus, HIV and mycobacteria and cryptococcus neoformans [56, 85–90] have been generated *in-vitro* and in some instances, cytolytic CD4⁺ T cells generated in culture, have been adoptively transferred to patients as part of an anti-tumor therapy [91, 92] or to control infections in stem cell recipients infected with EBV or CMV [93]. Also in mice, transfer of influenza-

specific, *in-vitro* generated CD4 CTL resulted in a reduced viral titer [94], even in the absence of CD8 or B cells, or before the primary response in normal mice could develop. This suggests that CD4 CTL might have active non-redundant roles in direct protective immunity. Perhaps more importantly, their MHC class II restriction could also be of particular importance in the control of pathogens such as the herpes viruses, EBV, CMV and especially HIV-1, that have developed sophisticated evasion strategies to avoid recognition by MHC class I restricted CD8 $\alpha\beta$ ⁺ T cells or NK cells. On the other hand, the fact that CMV has developed mechanisms to avoid the MHC class II pathway [95] could equally reflect the importance and active involvement of cytotoxic CD4⁺ T cells in anti-viral protective immunity.

One problem in understanding or appreciating the significance of CD4 CTL-mediated protection is the restricted MHC class II expression on target cells. Some viruses, such as EBV and LCMV can infect immune cells that constitutively express MHC class II on their cell surface [11]. However, many other viruses, such as CMV or influenza, target cells that normally do not express MHC class II molecules. Nevertheless, several published studies have shown that under inflammatory conditions, MHC class II can be drastically upregulated on various cell types including, β -cells [96], or on infected or IFN γ treated lung epithelial cells [97–99]. Also in humans, local upregulation of HLA MHC class II in various tissues seems to be a common consequence of inflammation [100, 101]. CD4 CTL might also function as bystanders and destroy infected MHC class II negative cells or other target cells indirectly through excessive production of IFN γ and TNF [102].

Recently, much attention has been paid on cytotoxic CD4⁺ T cells for tumor vaccination therapies as well. Indeed, not only infected but also malignant cells often induce MHC class II expression thus becoming CD4⁺ CTL target cells and strategies using anti-tumor specific CD4⁺ CTL are now beginning to be exploited. Studies of mouse γ -herpes virus 68 (MHV-68) have shown that CD4⁺ T cells can control herpes virus-induced malignancies *in-vivo*, independently of CD8⁺ T cells and B cells [103, 104]. Also in human, the growth inhibition of EBV-transformed B cells in healthy EBV carriers, was shown to be controlled in part by EBV-specific CD4⁺ T cells [105] and cytolytic CD4⁺ T cells, with specificity for various EBV epitopes expressed in the latent or the lytic phase of infection, have been isolated from PBMC of healthy donors [106]. Interestingly, EBV-specific CD4⁺ CTL have also been shown to directly target Burkitt's lymphoma, an EBV-associated B cell lymphoma, which resists recognition by CD8 $\alpha\beta$ ⁺ CTL due to down-regulation of the MHC class I antigen processing pathway [107]. In addition to MHC class II-based epitope vaccinations, also cell therapy approaches have been met with some success, including the treatment of EBV-associated Hodgkin's lymphoma and nasopharyngeal carcinoma using *in-vitro*-generated EBV-specific CD4 CTL. In some cases, complete remission was reached for a sustained period [56, 108], supporting a significant potent and long-lasting role for anti-tumor CD4 CTL *in-vivo*.

In addition to the potential direct participation of CD4 CTL, as direct protective immune cells to control infections and eliminate malignant cells, CD4 CTL themselves or the mechanisms that drive their differentiation, might also have regulatory capacity. Like conventional CD8 CTL, CD4 T cells with cytolytic functions, have the potential to modulate or control immune responses by eliminating MHC class II expressing professional antigen presenting cells, thus curtailing antigen presentation during priming and preventing further activation of naive T cells. Furthermore, the accumulation of CD4 CTL in the small intestine of recipient mice in the transfer model of induced colitis, correlated with less tissue destruction, suggesting that the CTL reprogramming resulted in less pathogenic effector cells compared to the inflammatory Th17 and Th1 effector cells that differentiated in parallel in this model [19]. Consistent with this, transfer of naive CD4⁺ T with forced

deletion of the *Thpok* gene in all activated CD4 T cells, resulted in increased differentiation of CD8 α -expressing CD4 donor T cells and less inflammation in the intestine of the *RAG*^{-/-} recipients [19]. In contrast, mice that received CD4 T cells with forced deletion of *Runx3*, showed accelerated wasting disease, which correlated with an increased IL-17 production [19]. Similarly, mice with a forced deletion of *Runx3* in T cells, showed more inflammation, with transmural infiltrates and epithelial cell damage, in response to a *Citrobacter rodentium* infection, whereas they cleared the pathogen more efficiently than wild-type mice. This was likely due to the greater frequency of IL-17-producing CD4⁺ effector cells in the absence of *Runx3* induction [19]. These observations suggested that, in addition to the promotion of the CTL program, *Runx3* might also counteract the Th17 pathway in activated CD4 T cells. Together, these results indicated that the reprogramming of CD4 T cells controlled by the gain of *Runx3* and the loss of ThPOK, not only leads to effector cells with direct protective capacity but the mechanism also deviate those cells away from becoming inflammatory Th cells, which have the capacity to cause tissue damage and other pathology.

Nevertheless, the increased accumulation of cytotoxic CD4⁺ T cells under chronic inflammatory conditions, such as auto-immune [109] and inflammatory bowel disease (IBD) [110], suggests that next to their potential beneficial protective functions, these cells might also participate in or contribute to inflammatory diseases. Indeed, whereas functional studies raised the possibility that disruption of this process may lead to uncontrolled CD4 Th responses, they also highlighted the potential of CD4 CTL to become potent pathogenic killer cells in the context of inflammatory cytokines, such as IL-15, which is excessively produced in IBD and Celiac disease or in virus-induced immune pathology. Although in general, most viral- or vaccine-induced CTL responses result in protective, sterilizing immunity for their hosts, they can also induce disease due to excessive or aberrant cytotoxicity. Virus control, as well as dampening of the inflammatory response, is important for minimizing pathology, however, whether an infection will result in severe or minimal tissue damage depends on numerous factors, not in the least on the inflammatory or (self)-regulatory potential of the responder T cells. For example, whereas normally, intra cranial (i.c.) LCMV infections in immune competent mice causes lethal meningitis and wasting disease mediated by excessive CD8 CTL responses, vaccinated immune competent mice elicit a CD8 CTL response that can either protect from immune pathology, or enhance immune pathology, depending on the potency and kinetics of the acute response [76]. Male mice show a low response and are not susceptible to immune-mediated pathology unless their responses have been boosted by vaccination. Female mice display much stronger responses and pre-existing immunity will protect them from immune pathology due to an effective and rapid CD8 CTL memory response that clears the virus before the immune response becomes excessive. Interestingly, in $\beta 2m$ -deficient mice, which do not have CD8⁺ T cells, i.c. LCMV infections still lead to lethality in female mice as well as in vaccinated male mice, strongly suggesting that CD4 CTL could develop into pathogenic effector cells. In agreement with this, in autoimmune diseases such as rheumatoid arthritis and ankylosing spondylitis, the amount of CD4⁺ T cells that display cytolytic features is related to the severity of disease, whereas treatment with anti-TNF, reduces their numbers [111–113]. Nevertheless, as of yet, there is no evidence that links the expanded CD4 CTL with self-antigen specificity. It is therefore also possible that the increase in CD4 CTL numbers associated with inflammation and autoimmunity is cytokine-driven or the consequence of an indirect bystander effect.

9. Conclusion and perspectives

Despite the irrefutable observations of CD4⁺ T cells with cytolytic capacity in conditions of viral infections, autoimmunity and cancers as well as their significant presence among the mucosal T cells that reside within the gut epithelium, we still lack convincing evidence that

CD4 CTL display important direct cytolytic functions *in-vivo*. Furthermore, continuous skepticism labeling these cells as an *in-vitro* artifact and doubting their physiological relevance has greatly overshadowed the need to investigate, track or target these cells as part of medical intervention strategies. Notwithstanding this, increasing evidence linking cytolytic CD4 T cells to the control of persistent viral infections and their noticeable inhibitory effects on malignant cell growth, have finally begun to turn the spotlight on them. More importantly, the new insights gained from the recent studies [4, 19], identifying these cytolytic CD4 effector cells as a true, separate T cell subset, marked by a unique network of transcription factors and the characterization of the mechanisms that drive their differentiation *in-vivo*, have opened up new avenues of research, to understand the biology and potentially pivotal roles of CD4 CTL in health and disease. The new appreciation of the functional potential of these cells has also created the necessity to rethink vaccine design and to aim for alternative strategies that also include CD4 CTL. Without a doubt, much progress will be made in the near future in improving medical intervention based on strategies that will exploit CD4⁺ CTL as an important target cell to prevent or treat infections, cancers and devastating autoimmune and other immune-related diseases.

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Highlights

1. The thymic CD4 Th-lineage commitment is not fixed and mature MHC class-II restricted CD4 T cells have the flexibility to reprogram to CTL.
2. The transcription factor, ThPOK, directs the Th-lineage fate of MHC class II restricted thymocytes and continues to suppress the Runx3-driven CTL program in mature CD4 Th cells.
3. Chronic antigen stimulation of mature CD4 T cells leads to the loss of the ThPOK controlled Th fate and the gain of the Runx3-driven CTL reprogramming.
4. CD4 CTL activated by their cognate antigen in the context of IL-15 become potent killer cells.
5. CD4 CTL are associated with chronic viral infections, malignancies and autoimmune disorders.
6. Chronic antigen-induced CD4 CTL have regulatory capacity.
7. The reprogram mechanism of CD4 T cells diverts them away from becoming pathogenic inflammatory effector cells.

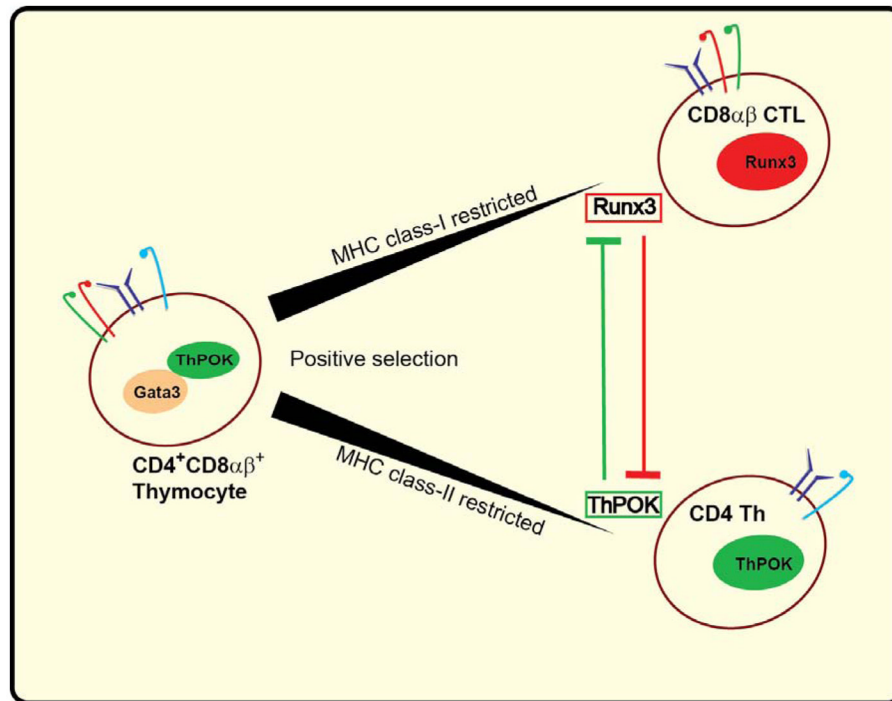


Figure 1. Th and CTL Lineage-commitment in the thymus

The reciprocal action and counter action of the master transcription factors, ThPOK and Runx3, drive the Th-lineage- and CTL-lineage commitment of MHC class II and MHC class I restricted thymocytes, respectively.

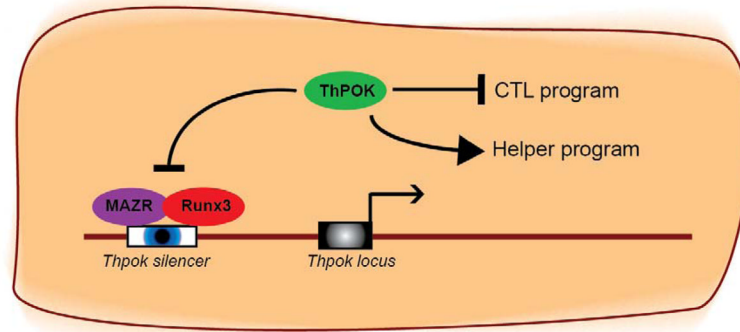


Figure 2. ThPOK is the master regulator of the CD4 Th-lineage fate

ThPOK drives the Th fate in developing thymocytes by counteracting Runx3. ThPOK continues to suppress the Runx3-controlled cytotoxic program in mature MHC class II restricted CD4 T cells. ThPOK controls its own expression by repressing the *Thpok* silencer in mature CD4 T cells. Derepression of the *Thpok* silencer results in the termination of *Thpok* transcription and loss of ThPOK expression and the activation of the Runx3-controlled CTL gene expression program.

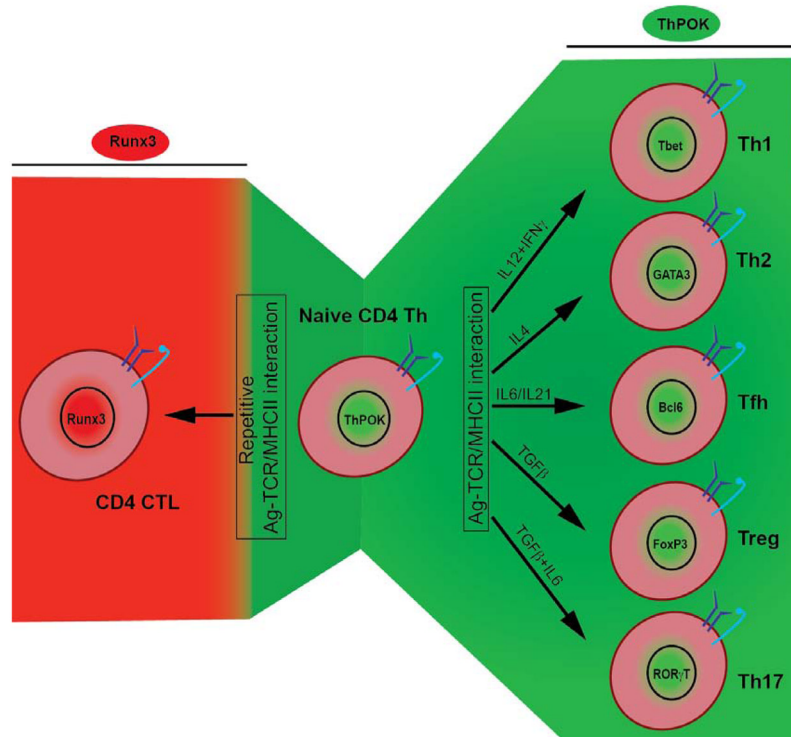


Figure 3. Functional plasticity of mature CD4 T cells

Antigen-stimulated CD4 T cells functionally differentiate into various Th subsets, driven by subset-specific transcription factors and cytokines. Repeated antigen stimulation leads to the loss of ThPOK expression and the induction of the Runx3-driven CTL polarization.