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Alternative memory in the CD8 lineage

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Abstract

A prominent population of innate $CD8^+$ T cells develops in the thymus of several gene deficient mouse strains, including *Itk*, *KLF2, CBP,* and *Id3*. These cells have the phenotype and function of memory $CD8^+$ T cells, without previous exposure to antigen. Surprisingly, the cytokine IL-4 plays a key role in their development. As this developmental mechanism was discovered, it came to light that innate $CD8^+$ T cells also exist in normal mice, and in humans. In this review we discuss how these cells develop, compare and contrast them to other CD8 memory cells, and discuss their potential physiological relevance.

"Innate T cell" is a term loosely used to describe distinct lineages of cells that develop in the thymus, which have a memory phenotype and upon T cell receptor (TCR) stimulation rapidly secrete large amounts of cytokines. These include cells that are restricted by MHC class Ib molecules that have limited tissue distribution and polymorphism, such as CD1d, Qa-1, H2-M3, and MR-1 [1–3]. Invariant NKT (iNKT) cells are the prototype of cells belonging to this family. Others include CD8αα intraepithelial lymphocytes, and mucosal-associated invariant T cells (MAIT cells) [4–7]. These innate T cells share several features in common, such as developmental dependence on IL-15, the SLAM associated adaptor protein (SAP) signaling pathway, and B7-CD28 interactions for functional maturation [8–10]. All of these subsets also share the property of having a highly restricted (oligoclonal) TCR repertoire, implicating specific self-antigens (lipid or peptide) in their development [4].

In 2006, a diverse population of polyclonal $CD8^+$ T cells was described in Tec kinase deficient mice, which shared functional and phenotypic similarity to innate T cells [11,12]. The ontogeny of these cells had been enigmatic until recently, when it was shown that IL-4 produced by NKT cells drives their development by inducing upregulation of Eomesodermin (Eomes). In this review, we summarize these recent findings and discuss the biological significance of innate CD8+ T cells.

Initial discovery of innate CD8+ T cells in ITK gene deficient mice

The Tec family of non-receptor tyrosine kinases are important components of the TCR signaling pathway [7,13]. Among the five Tec subtypes, genetic deficiency of inducible T cell kinase (Itk) was found to alter development of $CD8^+$ T cells in thymus and spleen [11,12]. In these mice, the majority of CD8 SP thymocytes had a $CD24^{lo}CD44^{hi}CD122^{hi}$ memory phenotype and rapidly produced IFNγ in response to TCR stimulation [11,12].

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These cells also expressed the T-box transcription factor Eomes [11], which is associated with expression of CD122 (IL-2 and IL-15 receptor β chain) and secretion of IFNγ in memory $CD8^+$ T cells [14]. However, unlike conventional memory T cells, these cells did not express T-bet. Because such cells resemble innate T cells in terms of their activated/memory phenotype and rapid production of cytokines, they were termed "innate $CD8^+$ T cells" [7,15].

Subsequent to these pioneering studies, a number of other mice deficient in T cell signaling molecules or transcription factors were shown to have elevated thymic innate $CD8^+$ T cells (Table I). These include the transcription factors kruppel like factor 2 (KLF2) [16,17], CREB binding protein (CBP) [18], and Inhibitor of DNA binding 3 (Id3) [19]. Mice with a mutation in Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP76:Y145F) [20] were also found to phenocopy the $Itk^{-/-}$ mice in terms of thymic $CD8^+$ T cells. All of the models listed in Table I were shown to have elevated Eomes in $CD8^+$ T cells in the thymus, arguably a hallmark of innate $CD8^+$ T cells. In all models, this was associated with a memory phenotype ($CD44^{hi}$ and $CD122^{hi}$) and ability to rapidly produce IFNγ when stimulated through the antigen receptor.

To date, it is unclear how all of these mutations result in a similar phenotype in $CD8^+$ T cells. The Tec family tyrosine kinases are required for full TCR-induced activation of PLC-γ, Ca^{++} mobilization, and Erk activation. A strong agonistic interaction between T cells and thymic epithelial cells was suggested to program immature thymocytes into T cells that have an innate phenotype and CD8αα co-receptors [32]. Therefore, it was proposed that reduced signal strength due to the lack of Tec kinase activity promotes the survival of T cells with high affinity TCRs, resulting in innate phenotype [7,13]. Indeed, the development of innate T cells in Itk-deficient mice was partially rescued by the hyperactive form of Erk [12]. Thus a reduced TCR signaling model could be consistent with altered CD8 lineage diversification. However, it was less clear how deficiency in the transcription factors CBP, Id3, or KLF2 would lead to similar phenotypes, particularly for KLF2, which is not expressed in DP thymocytes where Itk-dependent TCR signaling occurs [33]. Ultimately, clues to the initial discovery of the complex mechanism by which innate CD8+ T cells arise came from the experiments using mice deficient for KLF2.

Development of innate CD8 T cells is non-autonomous and requires IL-4

As described, CD8 single positive (SP) thymocytes from KLF2-deficient mice display a marked innate CD8 phenotype, with high levels of CD44, CD122, CXCR3, Eomes, and rapid production of IFNγ. KLF2-deficient mice also have profound peripheral T cell lymphopenia, as this transcription factor is required for T cell trafficking, and in its absence progenitors fail to emigrate from the thymus [34]. To distinguish autonomous and non-autonomous effects in KLF2-deficient mice, unequal mixed bone marrow chimeras were generated (Figure 1a) [16]. When KLF2-deficient bone marrow cells were transferred into irradiated hosts, together with a minority of WT cells, the WT "bystander" CD8 thymocytes adopted an innate CD8 T cell phenotype, similar to the KLF2-deficient thymocytes (Figure 1a, center panel). In contrast, when WT cells were the majority, neither population showed an innate CD8 phenotype (Figure 1a, right panel). These data demonstrate that innate CD8 T cell development in the KLF2-deficient mice is caused by extrinsic factors. Using the same strategy, the generation of Eomes-expressing innate $CD8^+$ T cells in the absence of Itk-, CBP- and Id3-mice was also found to be due to cell extrinsic effects [16,19].

The cytokine IL-4 is the extrinsic factor that causes innate CD8 development, because bystander cells that lack IL-4R do not upregulate Eomes, or show any other aspect of the innate phenotype, and they fail to produce IFNγ [16,19]. Furthermore, when KLF2-, Itk-, or

Id3-deficient mice were crossed to IL-4R-deficient mice, innate CD8 T cells did not develop [16,19]. IL-4 is known to induce Eomes in antigen stimulated $CD8^+$ T cells [35], and Eomes was required for the innate CD8 phenotype in KLF2-deficient mice, indicating that IL-4 exerts its effects via Eomes upregulation [17] (Figure 1b).

In the periphery, IL-4 stimulates naïve and memory $CD8^+$ T cells to proliferate in antigen-induced responses [36]. However, the bystander process occurs in the thymus, where IL-4 presumably acts on naïve CD8 progenitors. Indeed, when IL-4 was added during fetal thymic organ culture, it induced upregulation of Eomes in CD8 SP thymocytes [29]. Therefore, *Klf2*, *Itk* and *Id3* gene deficiency models clearly share a common mechanism whereby thymic overproduction of IL-4 results in the generation of innate $CD8^+$ T cells (Figure 1b and Table I).

PLZF+ NKT cells are the source of IL-4 that drives innate CD8 development

Because the innate CD8 phenotype was dependent on IL-4, it was of interest to determine why the *Klf2*, *Itk* and *Id3* deficiency models overproduce IL-4. As NKT cells can produce IL-4, it was intriguing that thymocytes in KLF2-deficient mice show an increased expression of promyelocytic leukemia zinc finger protein (PLZF), a key transcription factor involved in NKT cell development [37]. PLZF is a member of the BTB-zinc finger (BTB-ZF) protein family that controls a wide variety of cellular responses [38], including the proper development of αβ iNKT cells and γδ lineage cells expressing NK1.1, so called γδ NKT cells [21,39–41]. In the absence of PLZF, most thymic iNKT cells fail to fully differentiate, and remain in the $CD24^{hi}$ immature state. The reduced numbers of peripheral PLZF-deficient iNKT cells express high level of CD62L and lack specific functions, including preferential residence in non-lymphoid organs and rapid secretion of cytokines, especially IL-4 [40,41]. On the other hand, transgenic over-expression of PLZF in conventional αβ T cells induced homing to non-lymphoid tissue and rapid cytokine production [41,42].

Considering the crucial role of IL-4 for the generation of innate $CD8^+$ T cells in Itk-, KLF2- and Id3-deficient mice, and the fact that PLZF-deficient iNKT cells lose their ability to secret IL-4, it was hypothesized that $PLZF^+$ cells could be the major source of IL-4 in the thymus [16]. Indeed, mice deficient for KLF2, Itk, or Id3 have an expanded population of $PLZF^+$ cells, which are mostly αβ iNKT in KLF2-deficient mice [16] and γδ NKT cells in Itk- [21,43] and Id3-deficient mice [19,22,23] (Table II). Furthermore, double gene deficiency of PLZF together with Itk, KLF2 or Id3 led to the failure of innate CD8+ T cell development [16,19].

The accumulation of $PLZF^+$ cells in the thymus could be through enhanced differentiation, survival, or proliferation (Figure 2). The positive role of Itk and Id3 in TCR signaling cascades suggested that Itk- or Id3-deficient γδ T cells might have differentiated to PLZF-expressing cells instead of being negatively selected, due to reduced signaling strength [15]. CBP-deficiency or SLP76:Y145F mutation might lead to a similar process, as they have common defects in Itk-dependent genes after TCR stimulation [18,20]. As mentioned, however, KLF2 is unlikely to act at the stage where NKT are being selected, as it is not expressed in DP progenitors. KLF2 is known to control T cell migration by modulating cell surface receptor $S1P_1$ and CD62L [34,44]. Yet, altered cellular migration in KLF2-deficient mice is not likely to be responsible for the accumulation of $PLZF^+$ cells because $S1P_1$ deficiency does not induce an innate $CD8^+$ T cell phenotype [16]. Instead, a more likely hypothesis is that KLF2 deficiency might facilitate the survival or proliferation of $PLZF^+$ cells after selection. This model is consistent with previous findings that KLF2 is sufficient to program a quiescent phenotype in T cells by suppressing c-Myc expression [33], given

that c-Myc is critical for iNKT cell proliferation [45]. Therefore, it is possible that Itk, CBP and Id3 act at the selection stage of NKT cells from DP thymocytes and KLF2 regulates the proliferation of NKT cells after this stage (Figure 2).

Innate CD8+ T cells develop in BALB/c but not C57BL/6 mice

In the various gene deficient mice discussed, expanded PLZF$^+$ αβ or γδ NKT cells regulate the development of innate CD8$^+$ T cells in the thymus via production of IL-4. But does a similar pathway regulate CD8$^+$ T cells in normal mice? Interestingly, inbred strains of mice were shown to vary in their frequency of iNKT cells, with BALB/c mice on the high end of the spectrum, and C57BL/6 mice on the low end [46,47]. BALB/c mice have 3–5 times higher numbers of PLZF$^+$ cells in the thymus compared to C57BL/6 mice, with the majority of them being iNKT in BALB/c mice. Interestingly, CD8 SP thymocytes from BALB/c (but not C57BL/6) mice contain a distinct subpopulation of Eomes^{hi}T-bet^{lo}CD44^{hi}CD122^{hi} innate phenotype CD8$^+$ T cells that produce IFNγ. As in the various gene deficient models, this innate phenotype is dependent on IL-4 produced by NKT cells, because it is eliminated in BALB/c *Il4r*$^{-/-}$ and BALB/c *Cd1d*$^{-/-}$ mice [16]. Therefore, the developmental regulation of innate CD8$^+$ T cells by PLZF$^+$ population is not only a phenotype of some gene deficient mice, but also a physiological process in inbred mouse strains.

Innate CD8+ T cells in CIITA transgenic mice and an analogous pathway in humans

CIITA transgenic mice also produce an enlarged population of PLZF$^+$ CD4$^+$ T cells in the thymus [1,26]. CIITA is a transcriptional activator of MHC class II expression, and in CIITA^{tg} mice thymocytes express MHC Class II. This model system [27,28] was first designed to investigate the functional significance of CD4$^+$ T cells that could potentially develop by thymocyte-thymocyte interactions (T-T CD4$^+$ T cells), because human thymocytes, unlike mouse thymocytes, express MHC class II molecules on their surface [26,48]. Thus it was hypothesized that CD4$^+$ T cells could be positively selected by MHC II expressed on other thymocytes, which was confirmed in an in vitro reaggregate culture system [49]. In subsequent experiments using CIITA^{tg} mice, T-T CD4$^+$ T cells were found to have a striking similarity to iNKT cells, including IL-4 secretion upon activation, acquisition of memory markers [26], and developmental dependence on SLAM-SAP mediated signaling pathway [31]. Remarkably, about 30~40% of polyclonal CD4 SP thymocytes in CIITA^{tg} mice expressed PLZF (Figure 2), suggesting that T-T interactions during selection are critical for specifying an “NKT-like” T cell lineage, even when the restricting element was conventional MHC class II.

Interestingly, in CIITA^{tg} mice, almost all TCRα^{hi} CD8 SP thymocytes had CD24^{lo}CD44^{hi}CD122^{hi} memory phenotype with upregulated eomes and CXCR3 expression [29]. Similar to the described gene-deficient mice, and wild type BALB/c mice, increased numbers of CD8$^+$ T cells in CIITA^{tg} mice were normalized in CIITA^{tg} *Il4*$^{-/-}$ and CIITA^{tg} *Stat6*$^{-/-}$ mice, demonstrating that IL-4 and its signaling pathway are essential [27]. Of note, transgenic PLZF over-expression using a *Cd4* promoter failed to induce the development of innate CD8$^+$ T cells in wild type mice [29], indicating the potential need for additional signaling pathways or a specific PLZF gene dosage for IL-4 production in PLZF$^+$ cells.

The expression of HLA-DR in human thymocytes peaks during the fetal to perinatal stage and gradually decreases in postnatal thymocytes, and becomes virtually absent from 3~4 years old onwards [48]. The phenotype in CIITA^{tg} mice reflected that of human fetal thymocytes, in which up to 8% of CD4 SP thymocytes and 15% of splenic CD4$^+$ T cells expressed PLZF and up to 10% of CD8 SP thymocytes and 30% of splenic CD8$^+$ T cells

were positive for Eomes during the 2nd trimester of gestation [26,29]. The presence of a high percentage of CD122+CD161+ memory phenotype T cells in the fetus, which is generally considered to be free from gut microbiota or foreign antigenic challenge [50], suggests these cells are unlikely to be true memory cells, but to have developed through an alternative pathway (Table II). Although human thymocytes express MHC class II and SLAM molecules in the neonatal period, the newborn human thymus or cord blood contains few PLZF$^+$ CD4$^+$ T cells, or Eomes expressing CD8$^+$ T cells, the reason for which needs further investigation [26]. Nonetheless, it is interesting that MHC class II- and CD1d-dependent thymocyte-thymocyte interactions share a common thymic ontogeny in humans and mice respectively, and this suggests that IL-4 might be involved in generating Eomes-expressing CD8$^+$ T cells in humans as well.

What is the function of innate CD8$^+$ T cells?

Conventional memory T cells develop as naïve T cells in the thymus and become activated in the periphery by recognition of foreign antigen in an inflammatory context (i.e. infection) (Table II and Figure 3). Innate CD8$^+$ T cells phenotypically resemble memory T cells, yet do not require antigen experience to obtain this status, demonstrated by the fact that OT-I *Rag*$^{-/-}$ cells adopt a memory phenotype and function when present as bystander cells in KLF2-deficient mixed bone marrow chimeras [16]. In this regard, innate CD8$^+$ T cells resemble homeostatic (or virtual) memory T cells [51], which are generated in peripheral lymphoid organs in lymphopenic animals, in response to IL-7, IL-15 and self MHC-peptide [52,53] (Table II and Figure 3). On the other hand, innate CD8$^+$ T cells develop in the thymus in an IL-4 dependent manner (and presumably in response to self MHC-peptide). Are these 3 subsets of memory cells functionally equivalent? Certainly the fact that homeostatic and innate memory CD8$^+$ T cells do not require foreign antigen recognition for their generation means that that they are unlikely to play a critical role in secondary infections as do conventional memory CD8$^+$ T cells that were clonally expanded during a primary response (Figure 3). However, there is growing evidence for an “innate” role of memory CD8$^+$ T cells in primary infections, as sensors of an inflammatory environment [54]. For example, conventional memory OT-I T cells can produce IFNγ early on during infection with pathogens that do not encode the ovalbumin antigen [55]. This response could be induced by IL-12 and IL-18 produced by activated myeloid cells [54,56,57]. Furthermore, this response is protective, at least in the context where no other cells can produce IFNγ [55]. Both homeostatic and innate CD8+ memory cells also produce IFNγ in response to IL-12 and IL-18 [17,51]. Therefore it would seem most likely that homeostatic memory and innate CD8$^+$ T cells play roles early during infection, via production of IFNγ. In the human immune system, these types of non-conventional or unprimed CD8$^+$ T cells could be important as they are able to participate in host defense during the neonatal and early childhood period before conventional memory networks are established [1,16,26].

Whereas conventional memory CD8$^+$ T cells are composed of heterogenous subsets expressing T-bet and Eomes [58], innate CD8$^+$ T cells selectively express Eomes. There is a complex interplay between Eomes and T-bet in the generation of central and effector memory responses, with expression of T-bet being generally associated with good effector responses [14], and Eomes with long-lived memory responses. Therefore, the lifespan and role of innate CD8$^+$ T cells in the development of protective immunity remains to be investigated.

IL-4 is not typically associated with the generation of protective CD8$^+$ T cell responses. The ability of IL-4 to drive expression of Eomes, CXCR3, and IFNγ production in CD8$^+$ T cells is counterintuitive, given its role in CD4 helper responses, where it promotes a Th2 response, and suppresses a T-bet-mediated Th1 response [59]. However, the effect of IL-4

on $CD8^+$ T cells is not without precedent in the literature. Complexes of IL-4–anti-IL-4 drive the proliferation of $CD8^+$ T cells [36,60]. In fact, IL-4 is a potent growth factor for memory $CD8^+$ T cells at doses produced during normal immune responses [36]. In addition, IL-4 supports the proliferation [61] and conversion of naïve $CD8^+$ T cells into memory phenotype CD8 T cells in lymphopenic mice [62].

There is an important role for IL-4 in the development of $CD8^+$ T cell protective anti-malaria immunity, in which IL-4R deficient $CD8^+$ T cells specific for circumsporozoite protein of *Plasmodium yoelii* fail to develop into tissue-residing memory cells [63,64]. Interestingly, wild type BALB/c mice are much more effective than C57BL/6 strains at controlling malaria pathogens after immunization with radiation inactivated forms of *P. berghei* or P. yoelii sporozoites [65], which might be related to the high frequency of innate $CD8^+$ T cells in the BALB/c strain.

Concluding remarks

Until now, three types of $PLZF^+$ cells – αβ iNKT, γδ NKT and T-T $CD4^+$ T cells – have been identified in the thymus. Under certain conditions each of these can facilitate the development of innate $CD8^+$ T cells in mice and humans. Genetic evidence proved conclusively that IL-4 is required for this developmental effect. To date, it is not clear what stimuli cause NKT cells to produce IL-4 in the steady state. Interestingly, TLR signaling was recently shown to cause antigen-presenting cells (APCs) to present stimulatory self-lipids, through inhibition of α-galactosidase activity [66]. Perhaps endogenous signals can also cause α-galactosidase inhibition in thymic APCs, and these differ between inbred strains of mice. Regardless of the source, these findings highlight IL-4 as an important cytokine for the biology of memory CD8 T cells. Future work should focus on potential differences in the function of memory cells that are generated in an IL-4-dependent fashion, particularly as they are known to have skewed expression of T-box transcription factors. Since many pathogens, such as parasites, induce a strong IL-4 response, it will be interesting to determine how the CD8 response is qualitatively different in these infections compared to bacterial and viral infections that induce a primarily Th1 response.

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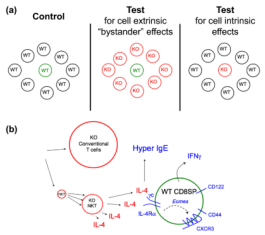

Figure 1. Innate CD8+ T cells develop via a cell-extrinsic mechanism

(a) Schematic of an "unequal mixed bone marrow chimera" approach wherein bone marrow cells are mixed at skewed ratios and used to reconstitute irradiated host animals. If a cell extrinsic bystander effect exists, wild-type (WT) progenitors in the minority would show a phenotypic change (middle). If the effect is cell intrinsic, gene-deficient (KO) progenitors in the minority would show a phenotypic change (right). These experiments showed innate CD8+ T cells develop via a cell-extrinsic mechanism. (b) NKT cells expand and secrete IL-4 in various gene-deficient mice. This induces wild-type "bystander" CD8 SP T cells to adopt memory phenotype and function. This effect of IL-4 on CD8 T cells is dependent on the transcription factor Eomes.

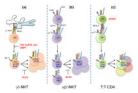

Figure 2. Various genes act at distinct stages to expand three types of PLZF^{+} cells

The genes indicated in red regulate the number of PLZF^{+} γδ NKT (a), αβ iNKT (b), or T-T CD4 T cells (c) in mice. ITK, SLP76, Id3 and CBP likely act downstream of the selection step that initiates γδ NKT development. KLF2 is more likely to regulate the later expansion of NKT, and affects both γδ NKT and αβ iNKT. The CIITA^{tg} creates MHC class II dependent thymocyte-thymocyte (T-T) interactions that generate polyclonal CD4^{+} T cells expressing PLZF.

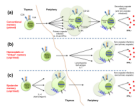

Figure 3. Multiple pathways to becoming memory-phenotype cells

a) Conventional memory cells are generated when naïve T cells become primed during infection via recognition of cognate antigens and inflammatory cytokines like IL-12 and IFNγ. Such cells upregulate T-bet and Eomes and facilitate the rapid clearance of pathogens during secondary infection, via cytolysis and production of IFNγ. b) Homeostatic or virtual (unprimed) memory cells are generated when naïve T cells are in lymphopenic conditions and respond to self-antigens and homeostatic cytokines like IL-7. Lymphopenic conditions can be induced by artificial radiation and acute or chronic infection. Such cells produce IFNγ in response to inflammatory cytokines IL-12 and IL-18, and thus could provide non-cognate or innate protection early on in infections. c) Bystander or unprimed memory $CD8^+$ T cells are generated when developing thymocytes respond to elevated IL-4, presumably together with self-antigen. Like homeostatic memory cells, bystander memory cells produce IFNγ in response to inflammatory cytokines and could provide non-cognate or innate protection early during infections. Bystander memory CD8+ T cells upregulate Eomes, but not T-bet, though the functional implications of this are not yet clear.

Table I

Multiple gene deficiency models give rise to innate $CD8^+$ T cells

Gene deficiency or transgenic model	Eomes expression in CD8 SP thymocytes	Cell extrinsic effect	Role of IL-4	Role of NKT lineage cells	References
Itk$^{-/-}$	elevated	yes	Increased IL-4R Hyper IgE Dependent on IL-4	Increase γδ NKT Dependent on PLZF and SAP	[10–12,16,21]
Klf2$^{fl/fl}$ *Cd4*^{Cre}	elevated	yes	Increased IL-4R Hyper IgE Dependent on IL-4	Increased αβ & γδ NKT Dependent on PLZF	[16,17]
Cbp$^{fl/fl}$ Lck^{Cre}	elevated	yes	Increased IL-4R	*n.d.*	[16,18]
SLP76:Y145F	elevated	*n.d.*[a]	*n.d.*	*n.d.*	[20,22]
Id3$^{-/-}$	elevated	yes	Increased IL-4R Hyper IgE Dependent on IL-4	Increased αβ and γδ NKT Dependent on PLZF and SAP	[19,22–25]
CIITA^{tg}	elevated	yes	STAT6 dependent	Increased PLZF expression in polyclonal $CD4^+$ T cells Dependent on SAP	[1,26–31]

[a] not determined

Table II

Different types of memory phenotype $CD8^+$ T cells

		Signals involved in generation		T-box transcription factor	Recall response	Innate function
		TCR	**Cytokines**			
Conventional (primed)		Foreign antigens	IL-2, IL-12, IFNα	T-bet Eomes	Yes	Yes
Non-conventional (unprimed)	Homeostatic memory	Self antigens	IL-7 (IL-15)	T-bet (?) Eomes (?)	No	Yes
	IL-4 memory (innate CD8s")	Self antigens?	IL-4 (others?)	Eomes	No	Yes