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REVIEW

Signals for antigen-independent differentiation of memory CD8$^+$ T cells

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Abstract

Conventional CD8$^+$ memory T cells develop upon stimulation with foreign antigen and provide increased protection upon re-challenge. Over the past two decades, new subsets of CD8$^+$ T cells have been identified that acquire memory features independently of antigen exposure. These antigen-inexperienced memory T cells (T_{AIM}) are described under several names including innate memory, virtual memory, and memory phenotype. T_{AIM} cells exhibit characteristics of conventional or true memory cells, including antigen-specific responses. In addition, they show responsiveness to innate stimuli and have been suggested to provide additional levels of protection toward infections and cancer. Here, we discuss the current understanding of T_{AIM} cells, focusing on extrinsic and intrinsic molecular conditions that favor their development, their molecular definitions and immunological properties, as well as their transcriptional and epigenetic regulation.

Keywords CD8$^+$ T cell · Antigen-inexperienced memory · Virtual memory · Innate memory

Introduction

T lymphocytes (T cells) are key players in cellular immune responses. Each T cell is equipped with a unique, clonotypic T cell receptor (TCR) capable of recognizing a number of small protein fragments (peptides) in complex with MHC molecules (pMHC) that are displayed on the surface of antigen-presenting cells. This ensures that, on a population level, T cells can respond to almost any infection. After a primary T cell-mediated immune response, a subset of antigen-specific T cells persists as long-lived memory T cells in lymphoid organs. Upon antigen re-challenge, memory cells are pre-programmed to differentiate rapidly into effector cells warranting effective secondary immune responses [1, 2].

In addition to these 'true' memory T cells (T_{TM}), other, non-conventional memory T cells are being identified and characterized, as documented by an increasing number of independent studies. Specifically, CD8$^+$ T cells with a memory phenotype can arise in an antigen-inexperienced manner, i.e., without previously being activated [3–5]. For simplicity, we here refer to these cells as antigen-inexperienced memory T cells (T_{AIM}). T_{AIM} cells constitute around 10–40% of total peripheral CD8$^+$ T cells, indicating their potential importance in the immune system [6–13]. Two major CD8$^+$ T_{AIM} cell types have been described: innate memory and virtual memory cells. Innate memory (T_{IM}) cells arise in the thymus, where they mostly depend on IL-4 [5, 14]. Virtual memory (T_{VM}) cells develop in the periphery and depend on IL-15 [15]. However, the distinction between and origin of the two types of T_{AIM} cells is still under debate [3, 16].

Functionally, T_{AIM} cells partially resemble conventional antigen-experienced T_{TM} cells by mounting a rapid immune response upon antigen stimulation [3, 5]. The immune response of T_{AIM} cells from young animals is particularly fast, suggesting that they provide extra protection in young animals when the immune system is not fully established [17]. In aged mice, T_{AIM} cells are major contributors to the anti-influenza response [18]. Besides mediating a TCR-dependent immune response, T_{AIM} cells have also been shown to provide antigen non-specific bystander protection in response to innate stimuli [8, 9, 19]. In addition, a potential role for T_{AIM} cells in the anti-tumor response has been

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suggested: T_{AIM} cells are able to infiltrate tumors and express high densities of the inhibitory receptor PD-1, a common target for immunotherapy [12, 20]. Furthermore, chemotherapeutic treatment of tumor cells increases the abundance of T_{AIM} cells [21]. These T_{AIM} cells can inhibit tumor growth in an MHC-I independent manner [21]. This unique responsiveness of T_{AIM} cells to different stimuli in combination with the substantial number of T_{AIM} cells warrants detailed investigation into this 'neglected' $CD8^+$ T cell compartment.

Another type of memory-phenotype cells are those induced by homeostatic proliferation in a lymphopenic environment [22]. It has been suggested that under lymphopenic conditions, these homeostatic proliferation-induced memory-phenotype cells (T_{HP}) arise from naïve T cells exposed to high levels of the homeostatic cytokine IL-7 [22]. The sum of these insights reveals that the T cell memory pool is very heterogenous and that the acquisition of T-cell memory features does not follow a single trajectory. Increasing our knowledge about T_{AIM} cells will help to better understand the role of these diverse memory T cell subsets in controlling specific immune responses. In this review we will not discuss T_{HP} (for a detailed review see Sprent et al. [22]), but focus on T_{AIM} cells that arise independent of experimentally induced lymphopenia. This review focuses on the origin of T_{AIM} cells and how their numbers and functions are regulated under specific physiological and immunological conditions. Furthermore, we will discuss transcriptional and (epi)-genetic regulation underlying the generation of T_{AIM} cells.

Markers of T_{AIM} cells

How are $CD8^+$ T_{AIM} cells identified and distinguished from T_{TM} cells? Several definitions of T_{AIM}, T_{VM} and T_{IM} are being used in the literature, defining the subsets based on surface markers, functionality and antigen-specificity [3–5]. The basic definition of a T_{AIM} cell is a T cell that has not been exposed to foreign antigen but has memory-like features. This antigen-inexperienced nature of T_{AIM} cells can be defined in several ways. One option is tetramer staining to identify $CD8^+$ T cells that are specific for defined pMHC class I complexes they have not been challenged with (e.g., the chicken ovalbumin OVA and vaccinia virus B8R epitopes) [9, 13, 15, 23–25]. Accordingly, one expects that $CD8^+$ T cells capable of binding the OVA and B8R tetramers are naïve, since they have not been previously challenged with the same pMHCs. Even though a single TCR can detect many different pMHCs, the presence of memory-phenotype cells among these tetramer-binding T cells in unchallenged mice indicates that these cells are not T_{TM} cells, but instead represent T_{AIM} cells. Another option to guarantee that cells are antigen inexperienced is to use $CD8^+$ T cells with a transgenic (tg) TCR, ideally on a background that prevents expression of endogenous TCRs, such as a RAG1/2 knock-out background [9, 25, 26]. When all $CD8^+$ T cells express the same tgTCR that only recognizes a synthetic peptide (e.g., the OT-I TCR recognizing the SIINFEKL epitope derived from OVA), the memory-phenotype cells identified without exposure to that specific antigen are T_{AIM} cells. Exposing the cells to the specific synthetic antigen will result in the formation of T_{TM} cells. To identify T_{AIM}-specific markers T_{AIM} cells have been compared to naïve (T_N) and T_{TM} cells. These markers are now broadly applied to determine whether memory-phenotype cells in unchallenged mice are T_{AIM} cells. Awareness of the T_{AIM} subset and the specific markers to detect them is important to determine whether memory-phenotype cells in unchallenged mice are T_{TM} or T_{AIM}. Unchallenged mice housed under specific pathogen-free (SPF) conditions typically have a memory-phenotype population, of which the majority (80–90%) are T_{AIM} cells [6, 27, 28]. Many studies in the past did not use T_{AIM}-specific markers and as a result were regarded all as memory-phenotype cells, including T_{AIM}, as T_{TM}. The current knowledge about the heterogeneity of the memory-phenotype population in unchallenged mice warrants a re-evaluation of our understanding of conventional T-cell differentiation [4]. Precise identification of the different memory subsets is thus required to gain further insight into the heterogenous process of memory T cell differentiation.

T_{AIM} cells are generally defined by several combinations of surface markers. They express many of the surface markers of conventional or true memory cells, including CD44 and CD62L. However, they can be distinguished from T_{TM} cells by low expression of CD49d, a marker of previous antigen experience [13, 15], high surface levels of CD122, part of the IL-2 and IL-15 receptor [13, 15, 29] and increased surface levels of CD5 [9, 30]. Furthermore, NKG2D, an activating cell surface receptor, is upregulated on a subset of T_{VM} cells, but not on all IL-4-induced T_{AIM} cells [9, 24, 31]. Besides these surface markers, the intracellular expression of the transcription factors T-bet and EOMES is also used to distinguish T_{AIM} from conventional memory cells. In C57Bl/6 mice, T_{VM} cells express high levels of both T-bet and EOMES, whereas T_{TM} are either T-bet^+ or $EOMES^+$ depending on being effector memory or central memory, respectively [26]. In BALB/c mice, T_{IM} cells both in thymus and periphery are T-bet^-EOMES^+ [14, 32]. In the thymus, T_{IM} cells are often described as $CD44^+CD122^+EOMES^+$ $CD8^+CD4^-$ thymocytes that express the chemokine receptor CXCR3 [14, 33] and have reduced levels of CD24, a feature of more mature $CD8^+$ thymocytes [34]. A subset of the peripheral T_{AIM} population also expresses CXCR3 [35]. Based on CD44 and CXCR3 expression, $CD8^+$ T cells can be divided into naïve (T_N; $CD44^-CXCR3^-$), intermediate ($CD44^+CXCR3^-$), and memory phenotype ($CD44^+CXCR3^+$) [31, 32]. Whether T_{IM} and T_{VM} are

different stages of one T_{AIM} subset or different subsets is still the topic of ongoing discussion [4, 16, 36]. It has recently been proposed that CD122 expression can function as marker to distinguish T_{IM} and T_{VM} in the periphery, with T_{IM} cells having lower expression of CD122 than T_{VM} but higher than T_N [11]. A detailed overview of T_{AIM} surface markers is provided in Table 1.

Mechanisms inducing differentiation toward T_{AIM}

The precise mechanisms that induce differentiation toward innate and virtual memory development are not completely understood, but two major components have been identified: quality of the TCR signal and cytokine signaling in the developmental niche.

TCR signaling in T_{AIM} cell differentiation

The quality of the TCR signal critically contributes to the decision making of developing and differentiating T cells. During thymic maturation, thymocytes expressing a TCR with very weak or strong affinity for self-peptides die by neglect or negative selection, respectively, and only thymocytes expressing intermediate affinity TCR are positively selected and migrate into the periphery [44]. In the periphery, T cells require tonic TCR signals for their survival [22]. Strong binding of an antigen to the TCR results in activation of the peripheral T cell and differentiation toward memory and effector cells [45, 46], but only when critical co-stimulatory signals are provided; otherwise the cells will become anergic, a long-lasting stage of T cell non-responsiveness [47]. The strength of TCR signaling in the thymus and periphery thus determines the fate of a T cell.

One characteristic of T_{VM} cells is their increased CD5 surface level [9, 30]. High CD5 serves as a proxy for high TCR affinity during thymic selection and negatively regulates TCR signaling in the thymus, thereby increasing the positive selection window by preventing negative selection [39–41]. The relative level of CD5 correlates with the percentage of T_{VM} cells in different mouse models [30]. Furthermore, CD5^{high} naïve cells acquire a T_{VM} phenotype more often after adoptive cell transfer than CD5^{low} naïve cells [9]. This suggests that cells with higher affinity for self-antigens or with higher TCR signaling are more prone to become T_{AIM}. This is corroborated in a mouse model with increased binding of the tyrosine kinase Lck to the CD8 co-receptor resulting in supraphysiological TCR signaling: mice with this increased TCR signaling have an increased number of T_{VM} [30]. The importance of signaling through the CD8 co-receptor is further demonstrated in a mouse model with constitutive expression of CD8αβ. This results in increased numbers of T_{IM} cells in the thymus, partly caused by extrinsic factors involving other thymic cell subsets and partly through cell-intrinsic mechanism [48]. Furthermore, mice deficient for DOCK2, which contributes to cellular signaling events by activating small G proteins, are more sensitive to TCR stimulation with low-affinity antigens and have increased numbers of T_{VM} cells [10]. This also suggests that increased sensitivity for tonic/self-peptide signaling induces T_{AIM} differentiation. A role for TCR quality was further supported by a study using mice with a transgenic TCRα and TCRβ chain. When these tgTCR mice are bred on a *Rag*-KO background, recombination of endogenous TCR genes is prevented and all T cells express the exact same TCR

Table 1 Markers of T_{AIM} cells

Marker	Function	Subset	Reference
CD122^{high}	Part of the IL-2 and IL-15 receptor [29]. Expression of CD122 is induced by EOMES and T-bet [37]. Lower CD122 expression in T_{IM} compared to T_{VM} [11].	T_{IM} and T_{VM}	[13, 15]
CD49d^{low}	α4β-Integrin; Part of the very late antigen-4 (VLA4) integrin that is involved in homing of effector T cells to the site of infection. CD49d is highly expressed on antigen-activated effector memory (T_{EM}) and effector (T_{EFF}) CD8$^+$ T cells [38]. CD49d upregulation is suppressed by IL-4 [38].	T_{IM} and T_{VM}	[13, 15]
CD5^{high}	Negative regulator of TCR signaling [39–41]. CD5 surface levels function as a proxy for TCR-binding affinity of the thymus [39–41].	T_{VM}	[9, 30]
NKG2D$^{low/high}$	Natural killer group 2 member D is a receptor expressed on activated CD8 T cells in mice and constitutively on CD8 T cell in human [42]. It functions as a co-stimulatory receptor [42]. NKG2D expression can be downregulated by IL-4 [31].	High in a subset of T_{VM}	[9, 24, 31]
CXCR3^{high}	Chemokine receptor involved in migration of T cells to peripheral site of infection [43].	T_{IM} and T_{VM}	[14, 31–33, 35]

(OT-I) with the same affinity. Under these Rag-deficient conditions, the percentage of virtual memory cells is low (< 5%) and remains low during aging [25]. However, $CD8^+$ T cells from mice on a Rag-proficient background, which are able to recombine the endogenous TCRα chain, generated higher T_{VM} cells at higher frequency and number, both of which increased with age (> 50%) [25]. This indicates that some TCRs are more likely to support the gain of a T_{AIM} phenotype than others.

The TCR repertoire of unchallenged T_{AIM} cells is distinct from that of naïve $CD8^+$ T cells (T_N) [12, 30]. Several TCRs have been identified as T_{AIM}-TCRs or T_N-TCRs, both in mice with an endogenous TCR and in mice expressing a transgenic TCRβ chain [12, 30]. Retroviral expression of transgenic T_{AIM}-TCRs contributes to an increased number of T_{AIM} cells in vivo [12, 30]. Cells expressing a T_{AIM}-TCR also respond stronger to stimulation with self-peptides by MHC-I on dendritic cells [12]. Furthermore, cells with a T_{AIM}-TCR are already $EOMES^+$ in the thymus and have increased surface CD5 expression, indicating that TCR-induced T_{AIM} differentiation already starts in the thymus [12, 20]. This increased TCR affinity also correlates with the higher convergence score reported for memory-phenotype ($CD44^+CD122^+$) cells [10]. The convergence score is the number of unique TCR sequences that encode for the same TCR and a higher score is indicative of selection by self-antigens [10]. Interestingly, co-culture of naïve $CD8^+$ T cells with chemotherapeutically treated tumor cells results in an increase of T_{VM}-phenotype cells independent of MHC-I [21], suggesting that other signaling pathways might overrule TCR signaling in certain conditions. Taken together, this data suggests a model in which T_{AIM} cells arise as a result of an intrinsically higher TCR signaling potential.

Given the increased TCR signaling in T_{AIM} cells, Drobek et al. studied if T_{AIM} cells are more likely to respond strongly to self-peptides and induce auto-immune pathology [30]. They found that T_{AIM} cells do not break self-tolerance and are less effective in inducing auto-immune pathology compared to T_{TM} cells [30]. This suggests that T_{AIM} differentiation might function as an escape mechanism for self-reactive $CD8^+$ T cells to avoid auto-immunity [30, 49]. Alternatively, when T_{AIM} cells recognize self-peptides, they might 'not properly' be activated/primed and as a result of this have reduced cytotoxic capacities compared to properly activated T_{TM} cells. In light of this, it is relevant to note that regulatory $CD8^+$ T cells also have a $CD122^+CD49d^-$ memory phenotype [50], although to our knowledge the immune-regulatory function of T_{AIM} cells has not been studied.

Cytokine signaling in T_{AIM} cell differentiation

Cytokine signaling is critical to ensure that activated T cells follow the right trajectory of differentiation. The two main cytokines involved in T_{AIM} development are IL-4 for T_{IM} and IL-15 for T_{VM} cells. IL-4 is mainly produced by invariant NKT (iNKT) and γδ-T cells in the thymus [14], although other thymic populations have also been reported as a source for IL-4 [51]. Mouse models in which the number of IL-4-producing iNKT cells, γδ-T cells or CD4 single positive T cells is increased have more innate memory cells [14, 52–55] as reviewed in [56]. Furthermore, the number of iNKT cells is higher in BALB/c mice compared to C57Bl/6 mice, correlating with a higher number of T_{IM} cells [14, 57–59]. When IL-4 signaling is reduced in mouse models with increased IL-4 production, either by ablating iNKT cells, deleting the IL-4 receptor or by blocking IL-4 with antibodies, the T_{IM} population is diminished, indicating that T_{IM} cells require IL-4 [53, 60]. On the other hand, in vivo treatment with IL-4/anti-IL4 antibody complex results in increased number of $CXCR3^+$ $CD8^+$ T cells that have increased EOMES expression, indicating that increased IL-4 levels contribute to T_{IM} differentiation [34, 61]. The exact mechanism of how increased IL-4 signaling stimulates T_{IM} differentiation is not known. It has been reported that IL-4 drives EOMES expression through Stat6- and AKT-dependent pathways [32, 62]. However, T_{IM} cells do not depend on increased IL-4 production under all conditions. There are also reports of T_{IM} cells arising independently of excess IL-4 production [63]. Taken together, IL-4 is important, and in most cases, required for T_{IM} differentiation in the thymus.

Although increased levels of IL-4 can contribute to an increased percentage and number of T_{VM} in the periphery [19, 23, 57, 64], IL-15 is the most important cytokine for peripheral T_{AIM} cells [3]. IL-15 is a key driver of homeostatic $CD8^+$ T cell proliferation under steady-state conditions and regulates memory effector functions [65]. Several lines of evidence reveal that in the periphery, T_{AIM} cells depend on IL-15. First, ablation of IL-15 production or abrogation of IL-15 signaling completely inhibits T_{AIM} differentiation and survival [9, 15, 66, 67]. Second, T_{AIM} differentiation in IL-15 depleted mice can be rescued by injecting IL-15/Rα complexes [9, 15]. Third, CD122, part of the IL-15 receptor complex, is more highly expressed on T_{VM} compared to naïve and T_{TM} [15]. Furthermore, a recent study suggests that regulatory CD4 T cells regulate T_{VM} differentiation by inhibiting IL-15 trans-presentation by $CD11b^+$ dendritic cells (DCs) [68]. How increased IL-15 signaling leads to the formation of T_{AIM} cells has not been fully described yet. It has been suggested that IL-15 signaling results in homeostatic expansion of T_{VM} cells that are already present in the periphery [15]. However, IL-15 signaling might also contribute toward T_{AIM} differentiation itself. The source of IL-15 for T_{AIM} cells are CD8α + DCs in peripheral lymphoid tissues [15]. This suggests that peripheral T_{AIM} mostly depend on naturally occurring IL-15.

T_{AIM} cell differentiation: a delicate balance of multiple signals

Taken together, the current understanding of signals involved in T_{AIM} differentiation suggests a model in which T_{AIM} differentiation is steered by a very delicate balance of at least two external signals mediated by the TCR and cytokine receptors and integrated intracellularly (Fig. 1). These signals lead to altered transcriptional output, which is further modulated by epigenetic mechanisms (see below). Natural fluctuations in gene expression of key genes that regulate TCR signaling or cytokine signaling or their downstream targets may render cells more or less susceptible to become T_{AIM} cells. This is in line with the current notion that fluctuations in gene expression occur over time within cell populations and that these fluctuations can have long term impact on features of the original cell and its offspring [69, 70]. Among the genes whose fluctuation might affect T_{AIM} differentiation is CD5, a negative regulator of TCR signaling. Fluctuations in CD5 expression may render cells more sensitive to tonic signals provided by self-peptide MHC complexes, making these clones more prone to inappropriate priming and differentiation, especially under pro-proliferative conditions like lymphopenia. In this context, the tyrosine kinase ITK, a mediator of TCR signaling, is also of interest. ITK plays an important role in T_{AIM} differentiation. ITK ablation in the T cell lineage results in increased numbers of T_{AIM} cells [66]. This skewed differentiation is IL-4 dependent and requires an external source of IL-4 [51, 56].

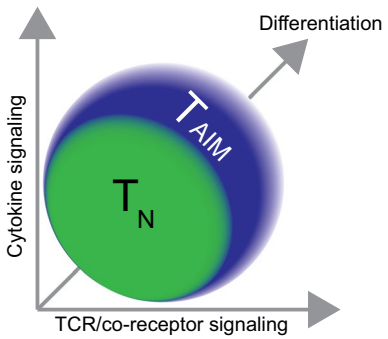

Fig. 1 Antigen-inexperienced memory T cell (T_{AIM}) differentiation: A delicate balance of integrated signaling. The quality of TCR/co-receptor and cytokine signals are central to T_{AIM} differentiation. Cytokines most important for T_{AIM} cells are IL-4 in the thymus and IL-15 in the periphery. Ablation of these cytokines or their receptors leads to a strong reduction in the number of T_{AIM} cells. The balance between cytokine and TCR signals determines whether a CD8$^+$ T cell remains naïve (T_N) or differentiates into a T_{AIM} cell. The TCR and cytokine signals lead to altered transcriptional output, which is further modulated by epigenetic mechanisms. The extracellular signals, and fluctuations in expression of genes involved in these signaling pathways due to transcription dynamics, as well as differentiation and proliferation together affect T_{AIM} differentiation. The net result is a dynamic population of T_{AIM} cells in mouse and humans

However, Huan and colleges suggest a cell-intrinsic role for ITK in T_{AIM} differentiation [51]. While in vitro TCR activation in combination with IL-4 results in reduced expression of the IL-4 receptor (IL-4R) and EOMES and a decrease in the percentage of T_{AIM} cells in WT CD8 single positive thymocytes, ITK-ablated CD8 single positive thymocytes are less sensitive to TCR activation and maintain higher levels of IL-4R and EOMES and a higher percentage of T_{AIM} cells [51]. This suggests that ITK tunes the response to IL-4 signaling cell intrinsically [51]. Given the role of ITK as a mediator of TCR signaling, TCR signaling might no longer lead to reduced IL-4 receptor expression on CD8 single positive thymocytes in the absence of ITK, thereby rendering the cells more sensitive to IL-4. Cross talk between TCR signaling and cytokine signaling is also shown by the effect of TCR signaling on IL-4-induced expression of EOMES in vivo, where IL-4-induced upregulation of EOMES is stronger upon weak TCR signaling compared to strong TCR signaling [62]. This all suggests a model in which a delicate balance of TCR signaling and cytokine signaling is required to keep cells in a naïve state or induce T_{AIM} differentiation.

Physiological regulation of T_{AIM} during development and infection

T_{AIM} cells are observed in unchallenged wild-type mice and also in humans. Increased numbers of T_{AIM} cells have been described in several genetically engineered mouse models (GEMM). The number of T_{AIM} cells is also regulated during normal physiological development and it changes during life. Most studies on T_{AIM} are performed in adult mice, which have a small but substantial population of T_{AIM} cells (~20% of total CD8$^+$ T cells) [6–13]. In young (infant) mice, there is a larger fraction of T_{AIM} cells (~35%) [23]. Using an elegant approach to time stamp T cells produced at different ages, Smith et al. mapped the fate of cells from different developmental origins [17]. They showed that in adult mice the CD8$^+$ T cells with a fetal origin have around five times more T_{VM} cells than CD8$^+$ T cells with an adult origin. This increased T_{VM} frequency in CD8$^+$ T cells with a fetal origin is not caused by lymphopenia, but rather is an intrinsic feature of CD8$^+$ T cells of early developmental origin [17]. Physiologically, the early-developed cells contribute more rapidly to an immune response compared to late-developed T cells, as shown by a rapidly increased number of early time-stamped cells at the beginning of the immune response. Early-developed T_{VM} cells respond in a more innate-like manner and differentiate predominantly toward short-lived effector cells, whereas late-developed T_{VM} cells give preferentially rise to long-lived memory cells [17]. This suggests that the T_{VM} cells developed during early stages of development might give increased rapid innate-like protection that is

needed in young animals with a developing immune system. Importantly, this data shows that the T_{AIM} pool is not homogenous, but that T_{AIM} cells developed during different stages are differentially programmed and have distinct functions.

In aged mice, the percentage and absolute number of T_{AIM} is again increased [9, 11, 28, 71]. The precise mechanism that contributes to this increase is unknown, but also in older individuals T_{AIM} cells depend on a combination of TCR and cytokine signaling. Aged mice that have a transgenic TCR but that are also able to undergo recombination of the endogenous TCRα have increased numbers of T_{AIM}, similar to younger mice [25]. This again demonstrates that the differentiation of T_{AIM} cells requires a specific level of TCR signaling. Furthermore, the surface levels of CD5 are increased in unchallenged $CD8^+$ T cells in aged mice [71], suggesting a selective advantage due to increased sensitivity for self-antigens. Consequently, in aged mice $CD8^+$ T cells expressing TCRs with high affinity for self-pMHC and strong signaling potential increase, favor T_{AIM} differentiation and with time T_{AIM} cells outgrow other $CD8^+$ T cells. Similar to T_{AIM} in young mice, T_{AIM} cells in aged mice still depend on IL-15, as was demonstrated by the absence of T_{VM} in aged IL-15 KO mice [6]. Furthermore, expression of CD122, part of the IL-15 receptor, is increased on a subset of T_{AIM} cells in aged BALB/c mice compared to young mice [11]. Taken together, this suggests that T_{AIM} cells that accumulate in older animals require similar TCR and cytokine signals as early-developed T_{AIM} cells.

Functionally, T_{AIM} cells are major contributors to an immune response against influenza in aged mice and gain a T_{CM} $CD49d^+$ phenotype afterward [18]. However, when aged T_{VM} and naïve $CD8^+$ T cells are transferred together, the T_{VM} cells are outperformed by the naïve cells [18]. Furthermore, aged T_{VM} are more monofunctional, secreting only one effector cytokine, whereas naïve cells are more polyfunctional, secreting several different cytokines [7]. Aged T_{VM} also have a reduced proliferative capacity [25] and they have a senescent phenotype characterized by resistance to apoptosis, increased γH2Ax indicating accumulated DNA damage and increased expression of Bcl-2 family members that control apoptosis [7]. The overall impaired immune response in aged individuals might among other changes also relate to reduced numbers of naïve $CD8^+$ T cells and increased numbers of later developed T_{AIM} cells.

There are several indications that the number of T_{AIM} cells is also actively regulated during specific immunological challenges. During helminth infection, the concentration of IL-4 in the thymus increases and this correlates with an increased T_{VM} pool [8, 19]. These newly expanded T_{VM} cells are able to mount a more effective immune response against lytic gammaherpesvirus infection [8] and bacteria [19]. Infection with trypanosomes results in cellular composition changes in the thymus, leading to increased production of IL-4 and IL-15 and an increased percentage of memory-phenotype cells in the thymus although these cells are $CD49d^+$ [72]. These T_{IM}-phenotype cells offer increased protection against trypanosome infection in an antigen-independent manner [72]. T_{IM}-phenotype cells ($CD44^+CXCR3^+CD49d^+$) are also seen after treatment with metformin, a drug used in treatment of type 2 diabetes, and they can contribute to increased protection against infection with *M. Tuberculosis* [73]. However, the origin of these metformin-induced T_{IM}-phenotype cells is not known [73]. Different infections can thus affect the immunological environment and thereby impact T_{AIM} differentiation. Increased numbers of T_{AIM} cells during certain immunological challenges are also seen in humans (discussed later); however, the exact mechanism is not always known. Given this recurrent correlation between infections and T_{AIM} development, further research into how infections promote the generation of T_{AIM} and the potential advantage of this increased T_{AIM} population in establishing immunity is crucial.

Most studies on T_{AIM} cells have been performed in mice housed under SPF conditions. This raises the question whether increased or decreased pathogen exposure affects T_{AIM} differentiation. Several studies have shown that T_{AIM} cells are also present in mice housed under germ-free conditions in a similar frequency as in SPF mice [11–13]. The presence of T_{AIM} cells in 'dirty' feral mice has only recently been investigated. Feral mice have an increased frequency of T_{AIM} cells compared to laboratory mice [11]. The frequency of T_{AIM} cells in feral mice is even higher than in F1 offspring of feral mice born in captivity [11]. This indicates that environmental factors may be involved in T_{AIM} differentiation, but the exact factors are not known. Co-housing of feral animals with laboratory animals affects the microbiome of the laboratory animals, but has very limited effect on T_{AIM} cells [11]. Further studies are thus required to determine which environmental factors might impact T_{AIM} differentiation.

Altogether, the number of T_{AIM} cells is not static but physiologically regulated. Both the age at which T_{AIM} cells are formed and the immunological challenges they encounter determine their number and function.

Transcriptional and epigenetic regulation of T_{AIM} differentiation

To better understand how T_{AIM} cells are regulated, we first need to define their developmental state. Are T_{AIM} cells fully differentiated memory T cells or do they represent a semi-differentiated subset? To answer this question, several studies have looked beyond the small number of surface markers detected by flow cytometry and determined the full transcriptome of T_{AIM} cells. Drobek et al. compared RNA-seq expression profiles of T_N

($CD44^-CD62L^+$), T_{VM} ($CD44^+CD62L^+CD49d^-$) and T_{TM} (K^b-$OVA^+CD44^+CD62L^+$, after infection with *Listeria monocytogenes*-OVA) [30]. Based on publicly available datasets of T_N and T_{TM} they defined naïve and memory gene signatures. T_{VM} cells are more enriched for memory signature genes than T_N, but less than T_{TM}. For naïve gene signatures T_{VM} are also in between T_N and T_{TM}. A similar intermediate phenotype is seen for expression of cytokines and chemokines [30]. This suggests that T_{AIM} cells indeed have an intermediate $T_N \rightarrow T_{TM}$ differentiation phenotype. However, this does not mean that they are just T_N cells that are only differentiated halfway. When compared with T_N and T_{TM}, T_{VM} cells have their own unique transcriptome features [7, 30]. This is shown by T_{VM} cells clustering away from T_N an T_{TM} based on principal component analysis or multi-dimensional scaling analysis [7, 30]. Taken together, T_{AIM} cells are a unique T cell subset with an intermediate differentiation phenotype as well as a unique transcriptome.

Further comparison of T_N and T_{VM} shows that T_{VM} cells have high expression of genes related to cell killing, inflammatory cytokines, granzymes, and genes related to NK cell receptors, and reduced expression of CD49d [7]. Furthermore T_{VM} cells have increased expression of genes involved in cytokine sensing, including IL-15 signaling mediators, and of the transcription factor EOMES [36]. A similar trend is seen for thymic T_{IM} cells, which are also enriched for cytokine receptors and memory signature genes compared to naïve CD8 single positive thymocytes [34]. These changes correlate with a strong increase in histone H3K27ac on the promotors of genes upregulated in T_{IM} cells. This histone mark is associated with active transcription and active enhancers [74]. The strongest epigenetic changes between T_N and T_{IM} are found in enhancers. Further comparison of H3K27ac peaks between T_{IM} and T_{TM} shows that many of the epigenetic changes in T_{IM} cells compared to CD8 single positive thymocytes are similar to the changes observed between T_N and T_{TM} [34]. However, not all changes observed in T_{TM} also occur in T_{IM} [34]. This suggests that T_{IM} cells use similar epigenetic programs as conventional antigen-experienced T_{TM} cells, but also have T_{IM}-specific changes in enhancer activity.

Interestingly, $CD5^{high}$ T_N cells ($CD44^-CD62L^+$), which are more prone to differentiate toward T_{VM} compared to $CD5^{low}$ T_N cells, already show upregulation of key T_{AIM} genes prior to memory differentiation [9]. $CD5^{high}$ T_N cells also already show increased expression of cytokine genes associated with homeostatic cytokine responsiveness (*Il2rb*, *Eomes*, *Il4r*) compared to $CD5^{low}$ T_N cells [9]. Furthermore, these cells show increased expression of gene clusters related to cell division and genes expressed in late effector and memory stages [67]. This upregulation of key T_{AIM} genes in T_{AIM} precursors seen in the peripheral $CD5^{high}$ cells, is in line with increased protein levels of EOMES in thymic T_{VM}- precursors [12]. These findings suggest that transcriptomic changes are not merely a result of having a more differentiated phenotype, but that they lie at the basis of antigen-inexperienced memory differentiation.

As mentioned before, T_{AIM} cells are a heterogeneous population, and this is also reflected in their transcriptome. Using the time-stamping technique Smith et al. isolated T_N ($CD8^+CD44^{low}$) and T_{VM} ($CD8^+CD44^{high}$) cells that developed during different time-points. Compared to T_{VM} cells produced during adulthood, T_{VM} cells produced early in life express more genes typically observed in short-term and late-effector $CD8^+$ T cells after in vivo stimulation [17]. This also correlates with their chromatin landscape, with early-developed T_{VM} cells having increased accessibility at binding sites for transcription factors associated with effector differentiation. On the other hand, transcription factors involved in repression of effector differentiation have increased binding in chromatin more accessible in late-developed T_{VM} [17]. This suggests that the differential regulome of T_{VM} cells with different developmental origins affects their responsiveness to key differentiation transcription factors [17]. T_{VM} from aged mice have a more senescent phenotype that correlates with increased expression of anti-apoptotic transcripts including *Bcl2* and downregulation of exhaustion markers, contributing to the accumulation of T_{VM} over time [7]. Thus, the developmental origin of T_{AIM} cells determines their transcriptome and epigenome. The transcriptomic and epigenomic data for T_{AIM} cells described here has been acquired by bulk sequencing of T_{AIM} populations. Given the heterogeneity of the T_{AIM} pool, single-cell sequencing will be important to further understand how T_{AIM} differentiation relates to regulation of the transcriptome and epigenomes.

Key epigenetic and transcriptional regulators in T_{AIM} differentiation

EOMES

One of the key transcription factors expressed in T_{AIM} cells is EOMES [36]. EOMES is a member of the T-Box family of transcription factors and is upregulated during conventional memory differentiation/antigen exposure [75]. EOMES is more highly expressed in T_{VM} developed in the fetal stage compared to T_{VM} developed during adulthood, but in all T_{VM} subsets its expression is higher than in T_N cells [17]. EOMES is very important in T_{AIM} differentiation, as is shown by the strong reduction of T_{AIM} cells in thymus and spleen in *Eomes*-KO mice [15, 53]. This was further confirmed in a model with transgenic overexpression of *Eomes*

in thymocytes, resulting in an increase in the percentage of T_{IM} cells [34].

EOMES is required for T_{AIM} differentiation at several levels. EOMES has been shown to bind directly to the promotor and internal regions of *Bcl2* and drive *Bcl2* expression (pro-survival protein), thereby giving memory cells a survival advantage compared to the strongly stimulated effector cells [76]. Besides driving pro-survival signals, EOMES is also involved in upregulation of CD122, which is part of the IL-15 receptor and upregulated in T_{AIM} cells [15, 29, 34, 37]. EOMES shows several interactions with other key regulators [34]. One potentially interesting interactor is RUNX3 because Runt motifs are slightly enriched in enhancer regions that have increased activity in T_{IM} vs T_N [34]. It has been suggested that EOMES is recruited toward regions where RUNX3 has bound [34]. In addition, BRG1, part of the SWI/SNIF chromatin remodeling complex, is critical to induce the EOMES-dependent program [34]. EOMES is thus a key transcriptional regulator in T_{AIM} cells that is embedded in a broader network of transcriptional and epigenetic regulators.

EOMES can be induced by several upstream factors including TCR stimulation and cytokines. IL-4 is one of the main drivers of EOMES expression in T_{AIM} cells. IL-4R knock-out (KO) mice show reduced EOMES levels in $CD8^+$ T cells, including T_{AIM} cells [32], while in vivo injection with IL-4 leads to increased EOMES expression in splenic T_{AIM} cells [61]. In vitro, IL-4 is also sufficient to induce EOMES expression in CD8 single positive thymocytes [62]. Interestingly, this induction is stronger under submaximal TCR stimulation [62]. Also, in vivo submaximal TCR affinity results in the highest EOMES upregulation and drives central memory formation [76]. Besides IL-4, type 1 interferons (IFNs) can also induce EOMES expression as is shown by the reduced EOMES levels in T_{AIM} cells lacking the receptor or signaling pathway required for type 1 IFN pathway. Loss of the type 1 IFN pathway correlates with a reduction in the number of T_{AIM} cells. Mimicking type 1 IFN signaling by PolyI:C results in increased EOMES levels and increased T_{AIM} numbers in vivo [77]. Several signals involved in T_{AIM} differentiation are thus also involved in upregulation of the master T_{AIM} transcription factor EOMES.

Epigenetic modulators

The epigenetic mechanisms involved in T_{AIM} differentiation are only starting to be unraveled, but several epigenetic factors have already been linked to T_{AIM} cells in different mouse models. The role of these epigenetic factors is mostly studied in conditional knock-out mouse models where the factor of interest is deleted in T cells during early T cell development. Among the epigenetic factors tested in this way is EZH2. EZH2 provides the catalytic part of the Polycomb repressive complex that is required for the generation of the repressive H3K27me3 mark [78–80]. Mice with conditional *Ezh2* KO in T cells have reduced H3K27me3 on the EZH2 targets, including the *Zbtb16* (PLZF) locus in iNKT cells. As a result, *Ezh2*-KO mice have aberrant iNKT differentiation and increased numbers of IL-4 producing iNKT cells, leading to increased numbers of T_{AIM} cells [81]. A similar mechanism is seen in mice lacking JARID2, a component of three lysine methyltransferase complexes that are involved in transcriptional repression, Polycomb repressive complex 2 (PRC2) that methylates histone 3 lysine 27 (H3K27), and the GLP-G9a and SETDB1 complexes that methylate H3K9. One of the targets of JARID2 is PLZF. Deletion of *Jarid2* results in reduced H3K9me3 on *Zbtb16* resulting in increased PLZF expression. This leads to an increase in IL-4 producing $PLZF^{high}$ iNKT cells and as a result thereof more T_{IM} cells in the thymus [33]. Thus, deletion of EZH2 or JARID2 contributes to T_{AIM} differentiation at least in part by affecting thymic IL-4 production. Since EZH2 and JARID2 are major epigenetic regulators that affect the many aspects of the epigenome, it is likely that there are also other genes differentially expressed in these mouse models that contribute to T_{AIM} differentiation. T cell-specific deletion of the histone acetyltransferase CREB binding protein (CBP) also results in increased T_{IM} cells in a cell-extrinsic manner [14, 82]. This has been shown by a mixed bone marrow chimera experiment. When bone marrow cells from WT and *Crebbp*-KO were co-injected into the same mouse WT cells acquired the same phenotype as KO cells, indicating that CD8 cell-extrinsic factors are involved [14, 82].

In contrast to the above-described epigenetic regulators that affect T_{AIM} differentiation in a cell-extrinsic manner, other epigenetic regulators impact T_{AIM} differentiation cell intrinsically. The histone methyltransferase DOT1L is responsible for methylation of H3K79, which is associated with active transcription in gene bodies [83, 84]. Deletion of *Dot1L* in the T cell lineage results in a strong increase in the number of $CD8^+$ T_{AIM} cells. This increase in T_{AIM} cells reflects a cell-intrinsic role of DOT1L as has been shown by mixed bone marrow chimeras [27]. When bone marrow cells from WT and *Dot1L*-KO were co-injected into the same recipient mouse, the WT cells remained mostly naïve, whereas *Dot1L*-KO cells still differentiated to T_{AIM} cells [27]. The onset of T_{AIM} differentiation in *Dot1L*-KO mice starts in the thymus where CD8 single positive thymocytes have increased expression of memory genes [27]. DOT1L-ablated T cells have aberrant expression of TCR signaling genes and reduced surface levels of the TCR complex [27]. This suggests that altered TCR signaling might be involved in T_{AIM} differentiation in *Dot1L*-KO mice. H3K79me2 in WT $CD8^+$ T cells marks active genes and loss of DOT1L activity results in downregulation of a subset of H3K79-methylated genes, whereas other H3K79-methylated genes

remain highly expressed [27]. What determines which genes are sensitive to loss of H3K79me2 is still unclear, but the downregulated H3K79me2 marked genes include genes encoding TCR, co-stimulatory components, and TCR signaling components [27]. *Dot1L*-KO CD8$^+$ T cells also show a group of upregulated genes that lack H3K79me2 in WT cells, suggesting that DOT1L controls expression of other transcriptional regulators, in particular repressors that indirectly regulate these genes. EZH2 was identified as a candidate negative regulator that is controlled by DOT1L and that can account for at least part of the indirect effects [27]. Taken together, DOT1L normally prevents T_{AIM} differentiation by controlling TCR signaling and expression, and by regulating a network of other transcriptional and epigenetic regulators. How DOT1L maintains this network, through its catalytic activity toward H3K79 or also by other functions, remains to be determined [85–88]. It would also be interesting to understand what the role is of putative H3K79 methyl 'reader' proteins and DOT1L binding partners in T_{AIM} differentiation, since these factors affect DOT1L output and activity [83, 89]. Finally, epigenetic regulators that impact DOT1L activity, including the histone deacetylase HDAC1 and the H2B ubiquitination machinery [83, 90–92], are also candidate factors affecting T_{AIM} differentiation.

Another epigenetic modulator that has a central role in T_{AIM} differentiation is the histone deacetylase HDAC7 [93]. HDAC7 a class IIa HDAC that functions as a transcriptional repressor [94]. The concentration of HDAC7 in the nucleus is actively regulated during T cell development in the thymus [95]. Deletion of *Hdac7* results in reduced numbers of iNKT cells and a general reduction in the number of thymocytes, but in an increased percentage of T_{IM} cells [93, 95]. Using mixed bone marrow chimeras, it has been shown that this increase in T_{IM} cells is cell intrinsic [93]. However, it is not clear how deletion of *Hdac7* results in increased percentages of T_{IM} cells. HDAC7 itself has very minimal deacetylase activity but performs most of its catalytic function through interaction with the co-repressive N-CoR/SMRT/HDAC3 complex [96–98]. In mature T cells, HDAC7 is continually phosphorylated resulting in translocation of HDAC7 from the nucleus to the cytosol [99]. In the thymus, HDAC7 is located in the nucleus during early thymocyte development and exported to the cytosol during positive selection [95]. The majority of the transcriptional changes observed during *Hdac7* deletion in thymocytes overlap with changes induced by positive selection [95]. This suggests that HDAC7 is a negative regulator of the downstream effects of TCR activation [95]. A possible mechanism for T_{AIM} differentiation in *Hdac7*-KO mice might thus involve increased TCR signaling. An overview of the role of epigenetic factors in T_{AIM} cells is outlined in Fig. 2.

Given the important role of epigenetic regulators in the execution of developmental transcriptional programs, it is likely that other epigenetic factors are also involved in T_{AIM} differentiation. Studying these regulators in conditional knock-out mouse models might prove difficult since other cells subsets are likely also affected in these mouse models. This is for example the case with EZH2. Upregulation of EZH2 target genes has been shown in *Dot1L*-KO T_{AIM} cells [27]. However, deletion of *Ezh2* in the T cell lineage has major effects on iNKT cell differentiation, thereby indirectly affecting T_{AIM} differentiation [81]. This strong effect of EZH2 on iNKT cell differentiation might mask the possible cell-intrinsic effects of *Ezh2* deletion in CD8$^+$ T cells. More advanced conditional ablation models combined with detailed epigenomic and transcriptomic analysis, preferably on single-cell level, will help to further understand the epigenetic mechanisms behind T_{AIM} differentiation.

T_{AIM} cells in humans

Most studies on T_{AIM} cells have been performed in mice. Recently some studies described T_{AIM}-phenotype cells in humans. Their precise origin and function are not known, but they are present in umbilical cord blood and fetal spleen, suggesting their antigen-inexperienced nature, and in blood and liver from mature adults [9, 54, 100–102]. In young adults T_{AIM} cells constitute around 5% of the peripheral CD8$^+$ T cell population and up to 15% in aged individuals [7, 103]. Human T_{AIM} cells are characterized by expression of the innate natural killer (NK) markers NKG2A or KIR and expression of the memory transcription factor EOMES [9, 101, 102]. Most putative human T_{AIM} cells in cord blood and in adult peripheral blood mononuclear cells have an effector memory re-expressing CD45RA (T_{EMRA}) phenotype [9, 102, 103]. Similar to T_{IM} cells in mice, human putative T_{AIM} cells most likely also arise in response to IL-4. This has been studied in the context of chronic myeloid leukemia, a condition where iNKT activity, a major source for IL-4 production, is reduced. The first indication for IL-4 being important for human T_{AIM} differentiation is that the percentage of T_{AIM}-phenotype cells is decreased in chronic myeloid leukemia, and that this decrease can be partially restored after complete remission of chronic myeloid leukemia [104]. Furthermore, stimulation with IL-4 in vitro results in an increased number of T_{AIM}-phenotype CD8$^+$ T cells [104]. The authors suggest that iNKT cells are the source of the T_{AIM}-stimulating IL-4 since the level of EOMES on T_{AIM}-phenotype CD8$^+$ T cells correlates with the level of PLZF on iNKT cells [104]. IL-15 has also been suggested to play a role in the maintenance/differentiation of human T_{AIM}-phenotype cells. KIR/NKG2A$^+$EOMES$^+$ T_{AIM}-phenotype CD8$^+$ T cells are preferentially expanded in HIV-infected patients [103]. Interestingly, HIV-infected

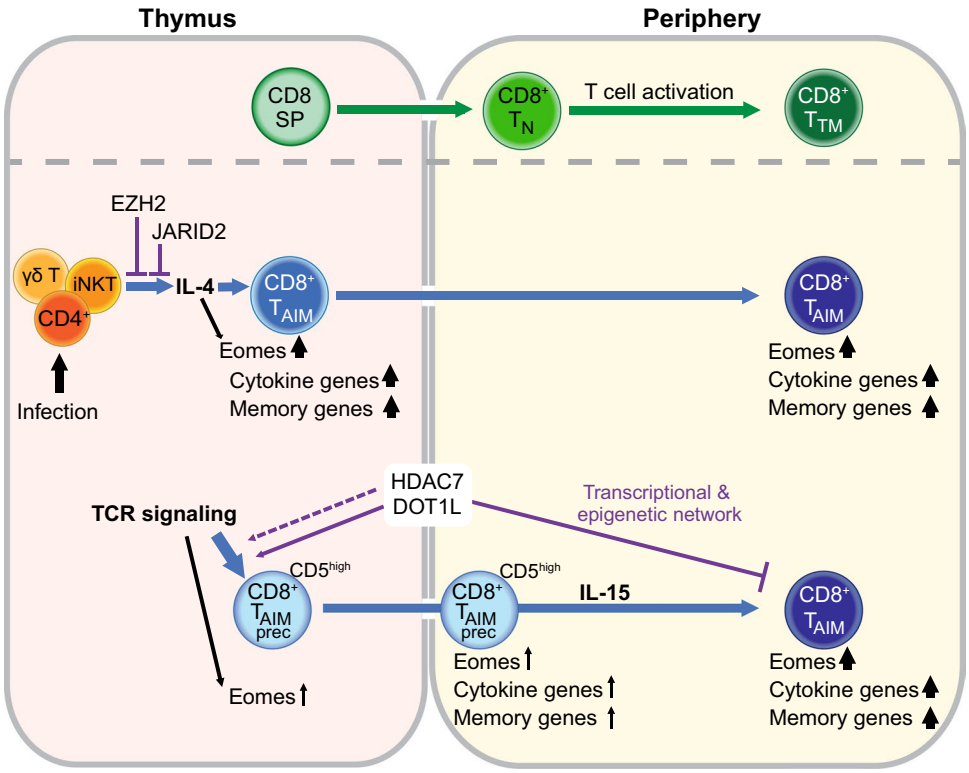

Fig. 2 A model of T_{AIM} differentiation regulators. During conventional $CD8^+$ T cell differentiation CD8 single positive (CD8 SP) thymocytes exit the thymus and enter the periphery as naïve (T_N) $CD8^+$ cells. Upon encountering a foreign antigen presented on the MHC class I complex of an antigen-presenting cells, T_N cells become activated and differentiate into short-lived effector and long-lived true memory (T_{TM}) cells. T_{TM} cells provide a rapid immune response when re-activated, thereby ensuring an effective secondary immune response. In addition to the conventional T_{TM} cells, memory-phenotype cells also arise in an antigen-independent manner. The differentiation of the antigen-inexperienced memory T cells (T_{AIM}) is guided by two main signals: cytokines and T cell receptor (TCR) signaling. Increased IL-4 levels in the thymus, either as a result of infection, or strain specific, or caused by deletion of the epigenetic modulator EZH2 or JARID2, induces T_{AIM} differentiation in CD8 SP thymocytes. These cells have increased expression of cytokine genes and memory genes, including the key transcription factor EOMES. EOMES expression can also be upregulated directly by IL-4. A specific level of heightened TCR signaling also affects EOMES expression and results in the upregulation of CD5 on naïve CD8 single positive thymocytes that are more prone to become T_{AIM} cells. Upon migration to the periphery, these $CD5^{high}$ cells already express mildly increased levels of cytokine genes and memory genes. IL-15 signaling further drives these cells to become T_{AIM} cells. The histone modifiers DOT1L and HDAC7 prevent T_{AIM} differentiation by regulating transcriptional and epigenetic networks that keep cells in a naïve state. Furthermore, DOT1L and, possibly also, HDAC7 are involved in regulating TCR signaling

untreated patients have increased concentrations of IL-15 in the lymph nodes and an increased population of bystander expanded memory-phenotype cells (defined as $CD45RO^+$ $CCR7^-$) [105]. The increase of T_{AIM}-phenotype cells in HIV-infected patients might also be (partially) due to increased homeostatic proliferation as a result of reduced $CD4^+$ T cell numbers, but this remains to be studied. These studies suggest that human T_{AIM}-phenotype cells are physiologically regulated and can be expanded under certain conditions.

As with murine T_{AIM} cells, the function of human T_{AIM}-phenotype cells has not been completely described. In vitro, the T_{AIM}-phenotype KIR^+/$NKG2A^+$ $CD8^+$ T cells rapidly produce IFNγ upon innate-like IL-12 + IL-18 stimulation [102, 104]. Furthermore, it has been suggested that T_{AIM}-phenotype cells may contribute to control of the HIV viral reservoir [103]. However, further research is required to validate the antigen-naïve state of these putative human T_{AIM} cells and to further study their origin and functionality.

Concluding remarks

Antigen-inexperienced memory cells form a substantial part of the $CD8^+$ T cell pool. They represent a unique memory cell subset with its own functions, transcriptome and epigenome. The number and function of T_{AIM} cells increases with age and during infections. This indicates that T_{AIM} cell formation play a dynamic role in the immune system. The mechanisms and dynamics of T_{AIM} differentiation have only been studied in the past few years and it is likely that many conditions that impact T_{AIM} differentiation and functionality

are yet to be discovered. For example, besides infections with helminths or trypanosomes, other infections that affect thymic IL-4 levels might also impact T_{AIM} differentiation.

The presence and heterogeneity of T_{AIM} cells shows that memory differentiation is not driven by a single process. Memory differentiation is guided by a complex network of different stimuli, involving TCR signaling and cytokines. Based on the conditions of their stimulation T_{AIM} cells have specific functional characteristics. On a transcriptomic level, T_{AIM} precursors already show increased expression of some key memory genes and fully differentiated T_{AIM} cells have their own unique transcriptome. These transcriptomic changes correlate with changes in the epigenome but further studies are required to fully understand how epigenetic regulators contribute to T_{AIM} differentiation by direct and indirect mechanisms.

In conclusion, CD8$^+$ T_{AIM} cells are a unique and intriguing subset of memory cells that help us to understand the complexity of memory cell differentiation and how different types of memory cells each contribute in unique ways to the immune response.

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Declarations

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