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Development of circulating CD4$^+$ T cell memory.

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Abstract

The ability of circulating $CD4^+$ T cells to retain memories of previous antigenic encounters is a cardinal feature of the adaptive immune system. Over the past two decades, since the first description of central and effector memory T cells, many studies have examined molecular mechanisms controlling $CD8^+$ T cell memory, with comparatively less research into $CD4^+$ T cell memory. Here we review a number of seminal studies showing that circulating memory $CD4^+$ T cells develop directly from effector cells; and in so doing, preserve features of their effector precursors. We examine mechanisms controlling the development and phenotypes of memory $CD4^+$ T cells, and provide an updated model that accommodates both the central and effector memory paradigm and the diverse T helper cell classification system.

Introduction

Immunological memory is a cardinal feature of adaptive immunity. The ability of immune cells, such as T cells to retain information from past pathogen encounters (or other immune challenges) allows the body to act more effectively during subsequent responses, thereby reducing the likelihood of severe disease. In cases of auto-immunity or immune-pathology memory T cells may also potentiate disease. Technological advances in molecular and cellular biology over the past two decades, for example in multi-dimensional flow and mass cytometry, RNA-sequencing, and epigenetics have provided an increasingly rich and diverse picture of what memory T cells are, how they might emerge, and what may influence their long term retention and function. Historically, research has focussed more on $CD8^+$ T cells than $CD4^+$ T cells, largely due to the relative ease in defining $CD8^+$ T-cell epitopes and making *in vivo* tracking reagents such as MHC-multimers and TCR transgenic T-cells. More recent concepts such as residency of memory T-cells in non-lymphoid tissues, were first cemented for $CD8^+$ T-cells. Increasing numbers of transcriptional and epigenetic regulators have been reported to control circulating and tissue-resident $CD8^+$ T-cell memory. In contrast, the molecular basis of $CD4^+$ T cell memory is less defined. Models for the development of tissue-resident memory $CD4^+$ T cells are in their infancy and are reviewed elsewhere[1]. Here, we synthesise observations from the last 20 years to provide an updated model for the differentiation of naive $CD4^+$ T-cells into circulating effector and memory states. This review focuses on assessing what is known, and what remains to be discovered about the development of circulating memory $CD4^+$ T cells.

What is a memory T cell?

It may be instructive to first outline a working definition of a memory T cell. A basic definition might be: a T cell previously activated via its TCR that exhibits a heightened capacity (relative to its naïve

precursor) for responding to cognate antigen. What exactly a "heightened capacity" entails is not strictly defined, but it can relate to the speed, magnitude or quality of the secondary response. To this basic definition, immunologists have added other more nuanced, but arguably less generalizable traits, such as persistence in the complete absence of antigen, existence at a higher frequency compared to naïve cells of the same specificity, and higher rates of proliferation than naïve cells[2]. While these extra traits are useful in many instances, they do not serve as absolute definitions for memory T cells. For example, memory $CD4^+$ T cell development occurs during persisting infections[3-5]. Evidence supporting a higher proliferation rate in memory T cells is not unequivocal, as memory T cells can display similar rates of cell cycle entry to naïve cells during recall responses[6]. Some memory $CD4^+$ T cells may exhibit lower production of certain cytokines (e.g IL-2), in favour of others[7, 8]. Thus, while there is no universal definition for memory T cells, in this review we focus on their capacity to mount a rapid effector response following secondary antigen exposure.

How do we identify and classify circulating memory $CD4^+$ T cells?

A memory T cell is best defined functionally, by its capacity to mount a recall response *in vivo*. However, it is not always possible to assess this experimentally. Another approach has been to identify and classify memory T cells via flow cytometry according to a set of cell surface protein markers, including CD62L, CCR7, CD45RA, CD44, CD45RO, CD27, CD127, PSGL1, Ly6C, CD69, CD103. Circulating memory $CD4^+$ T cells were initially classified by Sallusto *et al* into two main categories, as $CCR7^+$ $CD62L^{hi}$ $CD45RA^-$ central memory (T_{CM}) cells, and $CCR7^-$ $CD45RA^-$ effector memory (T_{EM}) cells, which were heterogeneous for CD62L expression[9]. T_{CM} cells have since been reported in various studies in experimental mice, to express higher levels of CXCR5, and lower levels of PSGL1 and Ly6C compared to T_{EM} cells[10, 11]. More recently, transcriptomic studies using microarrays and RNA-sequencing have allowed more detailed comparisons of these two memory T cell types. Such analyses have permitted inferences into how T cells might transition from naïvety to effector, from effector to memory states, and theoretically from one memory state to another. Taking into account also the phenotypic diversity that exists within $CD4^+$ T cell populations, it has been challenging to define unique protein markers for memory $CD4^+$ T cells. Are there in fact any genes that specifically control memory onset? If so, how might these proteins control transcriptional networks and epigenetic processes that promote and preserve memory? Given the substantial heterogeneity in effector $CD4^+$ T cell-types, what is the developmental and molecular relationships between these and memory subtypes?

The T_{CM} and T_{EM} paradigm.

The seminal study by Sallusto *et al.* described $CD4^+$ T_{EM} and T_{CM} cells in human peripheral blood, categorised by their migratory potential and their capacity to proliferate and produce cytokines *in vitro*[9]. Both memory cell-types exhibited enhanced sensitivity to TCR-mediated proliferation; yet T_{EM} cells

displayed a greater capacity for producing Th1 or Th2 effector cytokines compared to T_{CM} cells, which instead secreted only IL-2. This report revealed not only two types of circulating memory $CD4^+$ T cell, but also suggested that while T_{CM} cells were relatively homogeneous, T_{EM} cells varied in CD62L expression and their range of secreted effector cytokines. Reinhardt *et al.* reported similar findings when tracking antigen-specific $CD4^+$ T cells in the whole body of a mouse[12]. They characterised a circulating, non-lymphoid tissue resident, IFNγ-secreting memory $CD4^+$ T cell population, and a second lymphoid-resident population that produced IL-2, although lymphoid-homing markers were not examined[12].

More recently, there have been attempts to developmentally link T_{CM}/T_{EM} memory cells to the broad spectrum of effector $CD4^+$ T helper (Th) and regulatory subsets, including Th1, Th2, Th17, Th9, Th22, iTreg, Tr1 and follicular helper T cells (Tfh). These studies, which classify memory cells according to their Th-history, highlight that T_{CM}/T_{EM} markers appear insufficient for describing potential functions of memory $CD4^+$ T cells, despite being useful for considering trafficking potential[10, 13]. Given the broad spectrum of Th fates, it is perhaps unsurprising that two or three cell surface markers are incapable of accurately identifying different memory populations emerging from a pool of Th effector cells[14-16].

Memory $CD4^+$ T cell development

A central question in the field has been "*How do memory CD4⁺ T cells develop from naïve counterparts?*" In genetically tractable mouse models, a valuable approach was to indelibly mark, or fate-map, $CD4^+$ T cells that had up-regulated specific markers during their primary effector phase. Harrington *et al* employed an IFNγ fate-mapping mouse during LCMV infection to show that at least some of the Th1 effector pool gave rise directly to T_{EM} cells capable of a rapid Th1 recall response[14]. Similarly, Pepper *et al* fate-mapped CCR7 during *Listeria* infection, and found that $CCR7^+$ $CD4^+$ T cells, also expressing CXCR5, gave rise to T_{CM} cells[13].

Another approach in mouse models was to isolate $CD4^+$ T cells with specific flow cytometric phenotypes, adoptively transfer into an individual where unequivocal tracking was possible, and determine the resulting memory phenotype(s) [10, 11, 14]. Marshall *et al*, employed co-staining with P-selectin ligand 1 (PSGL1) and Ly6C during LCMV infection of mice, to cell-sort Th1 ($PSGL1^{hi}$ $Ly6C^{hi}$ or $Ly6C^{lo}$) and Tfh ($PSGL1^{lo}$ $Ly6C^{lo}$) cells, and found memory $CD4^+$ T cells with corresponding phenotypes[11]. Adoptive transfer suggested that Th1 effectors gave rise to Th1-poised T_{EM} cells, while $PSGL1^{lo}$ $Ly6C^{lo}$ memory cells, similar to Tfh cells, did not appear to make IL-2 upon recall *in vitro*[11]. Subsequently, Hale *et al* also used Ly6C, this time in conjunction with CXCR5, to assess Th1/Tfh biology in LCMV infection[10]. One again, $Ly6C^{hi}$ cells were observed to be Th1 cells (lacking CXCR5), while $Ly6C^{lo}$ cells expressed CXCR5 and were deemed Tfh cells[10]. Adoptive transfer of these subsets demonstrated a preference for Th1 cells to convert into Th1-like T_{EM} cells, and Tfh cells into CXCR5-expressing memory cells. The developmental link between effector and memory $CD4^+$ T cells was further suggested by bulk transcriptomics[10]. Together

with fate-mapping approaches, these reports support a direct developmental link between Th1 cells and Th1-like T_{EM} cells. Similarly, Tfh-like cells appeared to give rise to either or both T_{CM} and memory Tfh cells. However, these two developmental pathways were not necessarily devoid of overlap, since intermediate cell populations, PSGL1^{hi} Ly6C^{lo}, and CXCR5^{+} Ly6C^{int}, appeared to give mixed or different phenotypes after adoptive transfer compared to those with a clearer Th1 or Tfh phenotype. Therefore, although a powerful method, bulk adoptive transfer depends on the assumption that the sorted population is relatively homogeneous. In the cases outlined above, adoptive transfer of intermediate cell-phenotypes could give rise to erroneous conclusions about the multi-potency of an apparently homogeneous subset. To unequivocally assess effector to memory transitions in CD4^{+} T cells, methods for tracking single CD4^{+} T cells were required.

In a landmark single-cell tracking study by Tubo *et al.,* progeny from single naïve CD4^{+} T cells could differentiate during bacterial infection into more than one effector fate, preferences for which were influenced by TCR sequence and interactions with peptide:MHCII complexes[16]. Three fates were examined: CXCR5^{+} PD1^{+} , CXCR5^{+} PD1^{-} and Tbet^{+} CXCR5^{-}, which were termed GC-Tfh, Tfh and Th1 respectively. Given that T_{CM} precursors may comprise or exist within the CXCR5^{+} PD1^{-} population[13], cells referred to as Tfh in this study might also be classified as T_{CM} precursors. The ability of single naïve CD4^{+} T cells to give rise to both Th1 and Tfh cells was subsequently observed in our study of parasitic infection[17]. Tubo *et al* then employed single-cell tracking to explore memory, by longitudinally sampling splenic CD4^{+} T cells in mice by surgical biopsy. This elegant approach revealed that almost all effector CD4^{+} T cell clones yielded memory cells[15]. Notably, within each pool of memory cells, effector phenotypes (based on CXCR5 expression) were retained. Thus, fate-mapping, adoptive transfer and single-cell tracking approaches all support a model in which effector CD4^{+} T cells give rise directly to memory CD4^{+} T-cells. Moreover, there is consensus that Th1 cells give rise to T_{EM} cells poised for a Th1 recall response. Most infection models employed in this field exhibit a Th1 bias, meaning that generalisation across other T helper phenotypes, including Th2, Th17 and iTreg cells remains a work in progress[18, 19]. There is less clarity, at least in terminology, about what circulating CXCR5^{+} effector CD4^{+} T cells are: whether there is heterogeneity within this subset, and if so, whether different subtypes give rise to different memory states. Although flow cytometry, bulk and single cell transcriptomics suggest a strong Tfh signature in CXCR5^{+} effectors[10, 17], microscopy revealed heterogeneity in their location within T-cell zones, B-cell follicles, and red pulp areas in SLO. Current dogma suggest that some CXCR5^{+} effectors give rise to GC Tfh cells that could potentially develop into memory Tfh cells. However, this is difficult to assess without high-dimensional single-cell techniques since GC Tfh markers such as Bcl-6, ICOS, and PD1 appear to downregulate over time. It is likely that many CXCR5^{+} effectors, particularly those that do not substantially upregulate PD1, give rise to T_{CM} cells that retain stem-ness, functional fitness, and a capacity to develop into a spectrum of T helper phenotypes during recall[13]. Nevertheless, preservation of effector states occurs during memory onset[10, 13],

which has clear benefits assuming that effector $CD4^+$ T cell responses are both protective and non-pathogenic.

Controlling the development of $CD4^+$ T cell memory

The current model states that $CD4^+$ T-cell effector profiles are largely retained into memory. This predicts that the qualitative composition of the $CD4^+$ T-cell memory pool must be determined during the primary response, specifically when effector fate choices are made. The TCR sequence itself, and thus TCR interactions with peptide:MHCII complexes on conventional dendritic cells play an early role in controlling the choice between a Tfh/T_{CM} precursor fate or a Th1/Th2/Th17 effector fate[16]. If this were the primary determinant of effector fate choice, and given that it occurs prior to clonal expansion, one might expect all progeny from a naïve $CD4^+$ T-cell would give rise to only one progeny. This interpretation is not supported by the data, and in addition, tends to neglect the potential role of asymmetric cell division in fate choice[20]. Instead, single-cell studies revealed that the progeny of one naïve $CD4^+$ T cell frequently gives rise to both effector (e.g Th1 cells) and Tfh/T_{CM} precursors[16, 17]. Such heterogeneity could be triggered by asymmetric cell division as well as via complex interactions with peptide:MHCII complexes on different types of cDC, as revealed by intravital imaging [21].

Effector fate choice, and by extension, memory development, is not determined solely by an initial interaction with cDC. Reports in which MHCII:TCR interactions were blocked in a temporally restricted manner revealed that prolonged interactions with peptide:MHCII after initial priming supported both Th1 cell development[21], and long term retention of memory $CD4^+$ T cells[22]. Given that other myeloid cell derived signals[17, 23], including those proceeding via CXCR3 expressed on $CD4^+$ T-cells[23], or via ICOSL expressed on B-cells also influence effector fate choice[13], it is likely that activated $CD4^+$ T-cells are supported towards memory fates via cell-cell interactions with multiple cell types including cDC, monocytes and B-cells.

IL-2 is another signal that may influence $CD4^+$ T cell memory development. This cytokine has been reported to exercise its influence both during initial priming events, as well as at later check-points during effector phase responses[22]. $CD4^+$ T cells deficient in the high affinity IL-2 receptor alpha chain, CD25, exhibited a specific defect in Th1 effector responses during bacterial infection, but not T_{CM} or Tfh development [13]. Similar, though more modest effects were also seen in SLO during an allergic Th2 response in mice[19]. More recently, Snook *et al* linked CD25 expression with TCR signal strength, and suggested that higher TCR signalling strength triggered greater CD25 upregulation, skewing towards an effector Th1 phenotype[24]. However, adoptive transfer of $CD25^{hi}$ or $CD25^{lo}$ $CD4^+$ effectors into infection matched mice produced memory T cells with identical recall responses. This data provides a potential argument against a model in which there is a simple direct trajectory from effector cells to memory cells that harbour similar capacities to their effector precursors. Another recent study suggested that early IL-2 produced by nascent

193 Tfh/T_{CM} precursors signalled in a paracrine manner to $CD25^{hi}$ counterparts, which supported Th1
194 differentiation [25]. This study raises the possibility that local signals mediated by IL-2 between adjacent $CD4^+$
195 T-cells can influence fate, a theme explored *in vitro* by Polonsky *et al* [26]. In that study, IL-2 produced by a
196 quorum of neighbouring $CD4^+$ T cells triggered expression of CD62L, employed as a marker of T_{CM} cells[26] .
197 These findings appear to sit at odds with a model in which IL-2 signalling supports effector $CD4^+$ T cells
198 and T_{EM} cells, not T_{CM} cells. However, it should be noted that CD62L expression alone does not reliably
199 mark T_{CM} cells[9]. Together, these data provide strong support that early IL-2 signalling during priming
200 influences effector fate choice *in vivo*. In addition, McKinstry *et al* administered IL-2 late during viral
201 infection in mice, and protected effector $CD4^+$ T cells from apoptotic cell death during the contraction phase
202 of the response[22]. Moreover, signalling via CD27:CD70 appeared to trigger IL-2 production and support
203 progression to memory[22]. Thus IL-2-dependent mechanisms might act during effector to memory transition
204 to promote memory $CD4^+$ T cell survival.

205 An important question that remains to be answered concerns the massive contraction that effector
206 $CD4^+$ T-cells undergo during memory onset[27]. What determines whether an effector $CD4^+$ T cell dies or
207 progresses to memory? Is this process entirely stochastic, or is there a deterministic element to it? The study
208 from McKinstry *et al* points to specific molecular mechanisms, IL-2 and CD27/CD70 signalling, that
209 support effector $CD4^+$ T cells towards memory. A subsequent study by Shakya *et al* revealed that the
210 transcriptional co-activator, OCA-B, was critical for $CCR7^+$ $CD62L^+$ T_{CM} development, but played no role
211 in primary effector responses[28]. These two reports provide important evidence that T-cell extrinsic and
212 intrinsic mechanisms do exist, which influence not effector responses, but specifically effector to memory
213 transitions. Further research is required, therefore, to search for other moelcular mechanisms that
214 specifically control this process.

215 **Transcriptional and metabolic control of memory $CD4^+$ T cell development**

216 Next, we discuss the role of transcriptional regulators, epigenetic modifiers and metabolic programs
217 in contributing to gene expression programs in memory $CD4^+$ T cells. While many transcription factors have
218 been reported to influence memory development in $CD4^+$ T cells, including for example, NFAT, Bach2 and
219 Thpok, deficiency in these usually alters the primary effector response, understandably with consequences
220 for memory. Whether transcriptional regulators exist that specifically control effector to memory transitions
221 in $CD4^+$ T cells remains unclear. Given that memory T cells are transcriptionally quiescent relative to
222 effectors[29, 30], it remains an open question as to whether specific transcriptional networks are triggered to
223 elicit and preserve $CD4^+$ T cell memory. Evidence from Shakya *et al* provide functional support for this
224 concept[28], while Nish *et al* provide evidence that Tcf1 expression associates with self-renewal capacity in
225 memory $CD4^+$ T cells[20].

An important change in understanding of effector T cell differentiation has been an appreciation of the influence of chromatin structure[31, 32]. Changes in chromatin structure alter accessibility to transcription factors, which then promote or repress entire transcriptional programs. Therefore, examination of DNA modifications such as methylation, or histone modification and transcription factor binding, via chromatin immunoprecipitation followed by sequencing (ChIP-seq), have been useful alongside transcriptomic assessments to study T cells, for example in $CD8^+$ T cell exhaustion[33, 34]. While transcriptomics is particularly useful for studying changes in cellular state, such as effector to memory transition, quiescent cells such as naïve or memory T cells may be less open to this form of analysis. Instead, studies now highlight epigenetic remodelling as a process leading towards memory, as cells acquire distinct repressive or permissive chromatin states, "poising" them for future recall responses[35-37]. For example, targeted methylation profiling of ~2100 genes differentially expressed between naïve and memory $CD4^+$ T cells revealed differential methylation in 132 genes, with a subset of them displaying a correlation between gene expression and DNA methylation, as well as relevance to T cell function[38]. A more in-depth and comprehensive interrogation of the epigenome of human memory T cells in peripheral blood suggested progressive differentiation of epigenome and transcriptome, supportive of the linear model for memory development[39]. Importantly, this deep profiling of the epigenome allowed identification of novel gene targets associated with epigenetic signatures, including Foxp1, which influence transition to memory[39]. In studying epigenetics of memory commitment, Hale *et al.* found the *gzmB* locus displayed differential methylation between Th1- and Tfh-type memory cells[10]. Similarly, Szilagyi *et al.* showed that epigenetic programming allowed imprinting of gut homing receptor alpha4-beta7 expression that remained stable in memory T cells[40]. Given that the degree to which chromatin is open for transcription factor binding plays an important role in whether any given gene can be expressed in T cells, quantitative assessment of chromatin accessibility is likely to be extremely useful for the study of T cell memory. The advent of Assay for Transposase Accessible Chromatin using sequencing (ATAC-seq)[41] has already provided initial insights into human peripheral blood $CD4^+$ T cells[42, 43]. We anticipate that ATAC-seq profiling at bulk and single-cell levels will provide many new insights into memory $CD4^+$ T cells in humans and experimental animal models.

Changes in cellular metabolism during $CD4^+$ T cell differentiation suggested a possible role for these processes in influencing effector and memory T cell development. Naïve T cells change from being metabolically quiescent, surviving predominantly on pyruvate and fatty acid oxidation via oxidative phosphorylation (Oxphos), to a rapid increase in aerobic glycolysis to allow for rapid cell cycling upon TCR activation[44]. Following the contraction phase, memory T cells revert back to a naïve-like metabolic profile, characterised by an increased reliance on Oxphos[44]. However, memory $CD4^+$ T cells were also noted to have greater respiratory capacity[45] and elongated mitochondria due to Opa1-mediated mitochondrial fusion[46]. As TCR-signalling activates metabolic pathways [47], recent studies have unveiled roles for metabolic regulators

in driving effector fate differentiation[48]. Increased saturated fatty acid oxidation promoted preferential differentiation towards a non-lymphoid homing T_{EM} phenotype[49]. This was associated with augmented surface TCR clustering and increased TCR-inducible PI3k/Akt signalling. Oestreich *et al.* showed that Bcl6 directly repressed target genes of the glycolysis pathway[50]. In contrast, T-bet, which antagonizes Bcl6, was required to sustain glycolytic activities in Th1 cells, thus providing a link between transcriptional regulation, metabolic program and T cell "fate" choice. Given that these reports focus on effector $CD4^+$ T cells, future research will likely examine specific metabolic requirements for effector to memory transition, and memory $CD4^+$ T cell longevity.

A model and concluding comments

A model proposed in 2011 by Pepper and Jenkins has endured[51]. Here, we provide an update to this model based on more recent data (Figure 1). Naïve $CD4^+$ T-cells, primed by conventional dendritic cells in secondary lymphoid tissue, are influenced before, during and after clonal expansion towards either 1) an effector fate including Th1, Th2, Th17 or iTreg cells or 2) a separate lineage comprised of Tfh cells and/or T_{CM} precursor cells. The early innate and pro-inflammatory cytokine milieu determines the type of effector fate choice, as well as whether Tfh and T_{CM} precursors acquire a Th1, Th2, or Th17 "flavour". The influence of TCR-signalling strength and IL-2 on effector fate starts before clonal expansion. Subsequent cell-cell interactions with $MHCII^{hi}$ myeloid cells, and $ICOSL^+$ B-cells also influence effector fate choice. At peak effector phase, apoptotic cell death induces a dramatic contraction in effector numbers, with specific soluble signals, cell surface ligand interactions and T-cell transcriptional circuits allowing a subset of effectors to avoid apoptosis. Survivors of the effector lineage give rise to T_{EM} cells, while T_{CM} and possibly memory Tfh derive from the other lineage.

In conclusion, numerous lines of evidence support direct transition of effector $CD4^+$ T-cells to memory. However molecular mechanisms by which $CD4^+$ T cells transition from effector to memory states are only now beginning to emerge. We know even less about molecular processes that might be required to maintain circulating $CD4^+$ T-cell memory for extended periods of time. Finally, although tissue-resident memory $CD4^+$ T-cells were not discussed here, it will be useful to determine how these cells are developmentally related to effectors and circulating memory cells. To address these knowledge gaps, and given that memory $CD4^+$ T cells are usually present in very small numbers, newer technologies such as ultra high-dimensional cytometry and single-cell multi-omics approaches will be informative in the future.

LEGEND

FIGURE 1: A model for development of circulating CD4$^+$ T cell memory. Naïve CD4$^+$ T-cells, primed by cDC, are influenced towards either an effector fate including Th1, Th2, Th17 or iTreg cells, or a separate lineage comprised of Tfh cells and/or T_{CM} precursor cells. Innate cytokines determine the type of effector fate choice, as well as whether Tfh and T_{CM} precursors acquire a Th1, Th2, or Th17 "flavour". TCR-signalling strength and IL-2 influence effector fate early. Interactions with MHCII^{hi} myeloid cells and ICOSL$^+$ B-cells also influence effector fate. At peak effector phase, apoptotic cell death induces contraction in effector numbers, with soluble signals, cell surface ligand interactions and T-cell transcriptional circuits allowing a subset to avoid apoptosis. Survivors of the effector lineage give rise to T_{EM} cells, while T_{CM} and possibly memory Tfh derive from the other lineage.

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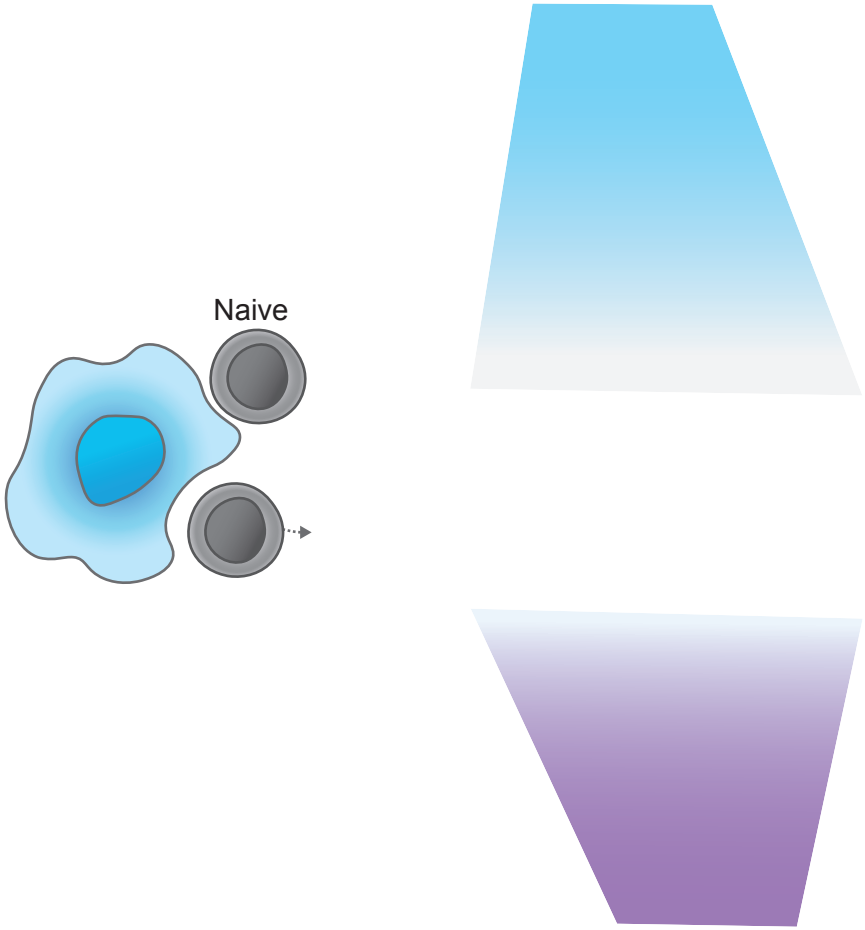
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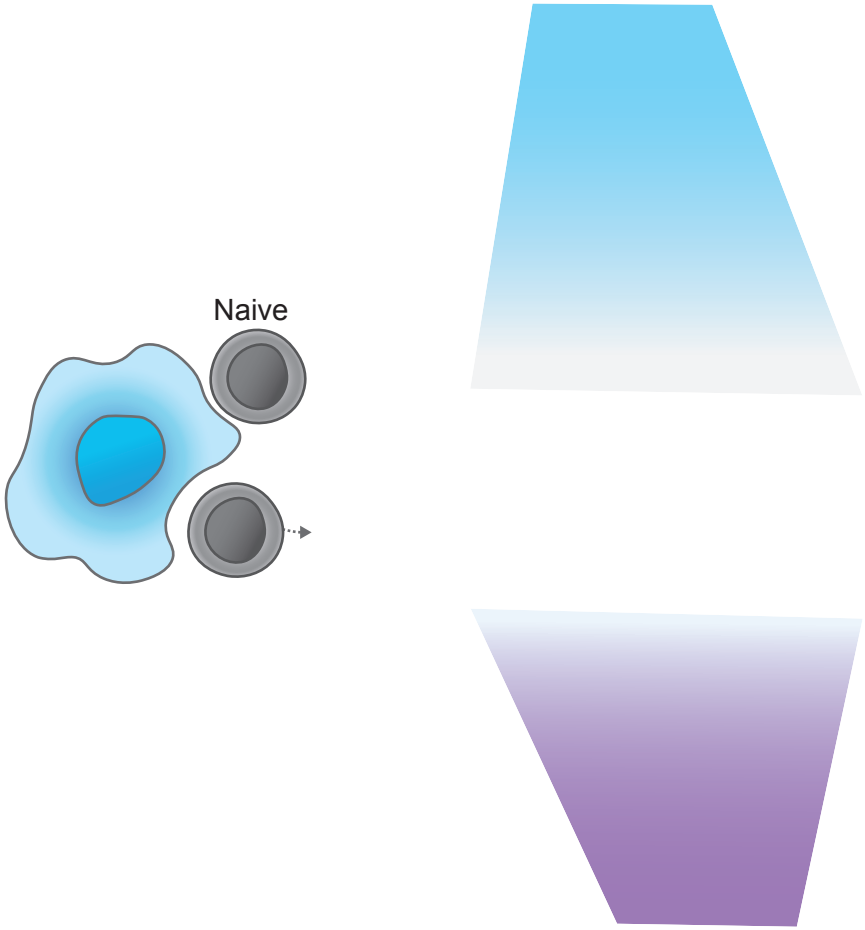
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