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Review

The who's who of T-cell differentiation: Human memory T-cell subsets

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Following antigen encounter and subsequent resolution of the immune response, a single naïve T cell is able to generate multiple subsets of memory T cells with different phenotypic and functional properties and gene expression profiles. Single-cell technologies, first and foremost flow cytometry, have revealed the complex heterogeneity of the memory T-cell compartment and its organization into subsets. However, a consensus has still to be reached, both at the semantic (nomenclature) and phenotypic level, regarding the identification of these subsets. Here, we review recent developments in the characterization of the heterogeneity of the memory T-cell compartment, and propose a unified classification of both human and nonhuman primate T cells on the basis of phenotypic traits and in vivo properties. Given that vaccine studies and adoptive cell transfer immunotherapy protocols are influenced by these recent findings, it is important to use uniform methods for identifying and discussing functionally distinct subsets of T cells.

Keywords: Flow cytometry · Human T-cell subsets · Memory T-cell differentiation · Naïve T cells

Introduction

Following positive and negative selection, T cells are released from the thymus as mature, naïve T (T_N) cells harboring a given epitope specificity. In response to cognate antigen (Ag) encounter, T_N cells proliferate and differentiate into effector cells, the vast majority of which migrate to peripheral tissues and inflamed sites to facilitate destruction of infected targets (reviewed in [1]). Following Ag clearance, such as that in smallpox vaccination, >95% of the effector cells die while a small pool of T cells ultimately develops into long-lived memory T cells [2].

The development of widely applicable technologies, i.e. monoclonal antibody production and flow cytometry, has provided a unique contribution to immunology. Together, monoclonal antibodies and flow cytometry have allowed the phenotypic interrogation of single cells within a heterogeneous cellular population, as well as the identification and viable isolation of discrete cell populations from a fluid or tissue. Currently, fluorescence-based flow cytometry can simultaneously detect 18 different markers on the same cell (reviewed in [3]). Recently, cytometry time-of-flight (CyTOF) technology, using isotope-labeled antibodies and measured by mass spectrometry, expanded this capability to 34

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different parameters [4], further revealing the heterogeneity of memory T cells (reviewed in [5]). Dozens of subsets, expressing unique combinations of surface and intracellular markers, and with distinct cellular functions, can be identified and enumerated in this manner [6]. Given this vast heterogeneity, a consensus has still to be reached on the phenotypic definition of T_N cells and various memory T-cell subsets as well as on their nomenclature. Currently, the different combinations of markers used to define such cells complicate comparability between studies. This review illustrates the memory T-cell compartment's complexity according to the phenotypic, functional, and persistence properties of discrete memory subsets. We propose a unified model of human memory T-cell differentiation along with a set of canonical markers to be used for their delineation.

Subsets of naïve and memory T cells

The historical path to T-cell diversity

In the 1980s, multiple researchers suggested that human T_N and memory cells could be separated on the basis of differential expression of surface molecules, including CD45R (now CD45RA), LFA-3 (CD58), and LFA-1 (then CDw29, now CD11a) or binding to the monoclonal antibody UCHL-1 (recognizing CD45R0) [7]. CD45RA and CD45R0 correspond to high and low molecular weight protein products of splice variants of the CD45 gene, respectively [8, 9], with CD45R0 being preferentially expressed on memory cells [10, 11]. $CD45R0^+$ cells are found infrequently in neonates [12] and progressively accumulate with age [13].

The analysis of homing receptors revealed that T cells are heterogenous in their capability to localize to peripheral tissues: in particular, T_N cells express high levels of the lymph-node homing receptor CD62L (L-selectin), while memory cells are split into $CD62L^+$ and $CD62L^-$ subsets [14]; the expression of mucosa- and cutis-associated homing Ags is confined to the $CD62L^-$ subset [14]. The mutually exclusive expression of CD103 and CLA suggests exclusive homing potential of T cells to the gut and skin, respectively [15]. These reports led to hypothesize that T-cell memory is organized in subsets that are selectively distributed in the body according to their effector functions.

Central and effector memory T cells defined by function

The development of techniques for measuring cytokine production at the single-cell level allowed detailed correlations between the functional properties of T cells and their phenotype [16]. For example, CD27, a member of the tumor necrosis factor (TNF) receptor superfamily, and C-C chemokine receptor 7 (CCR7), both mediating homing to the LNs, have been used in combination with CD45RA to phenotypically define subsets of $CD8^+$ memory T cells on the basis of their effector functions [17, 18]. $CD45RA^-$ $CD8^+$ memory T cells expressing CD27 produce both IL-2 and IFN-γ but lack immediate killing activity (therefore termed "memory"), while $CD45RA^-$ $CD8^+$ T cells that are $CD27^-$ produce mostly IFN-γ and TNF but not IL-2 and are capable of immediate cytotoxicity ex vivo (hence termed "effectors") [17]. Similarly, both $CD4^+$ and $CD8^+$ memory T cells that express CCR7 produce high amounts of IL-2 but low levels of other effector cytokines (e.g. IL-4, IL-5, and IFN-γ), while $CCR7^-$ memory T cells produce high levels of IL-4 and IL-5 ($CD4^+$ T cells only), and/or IFN-γ (both $CD4^+$ and $CD8^+$ T cells), and contain preformed perforin granules for immediate cytotoxicity [18]. $CCR7^+$ memory cells were named central memory (T_{CM}) cells because of their potential to home to secondary lymphoid tissues [18]. Their $CCR7^-$ counterparts were named effector memory (T_{EM}) cells because of their rapid effector function ex vivo and their potential to home to peripheral lymphoid tissues [18]. A precursor–product relationship between T_{CM} and T_{EM} cells has been suggested by the fact that T_{CM} cells retain longer telomeres than T_{EM} cells and are capable of generating T_{EM} cells in vitro, but not vice versa [18].

A plethora of studies in mice, nonhuman primates (NHPs) and humans later confirmed the concept that T_{CM} cells are early differentiated progenitors, can self-renew and, simultaneously, generate more differentiated progeny [19–21]. T_{CM} cells were thus thought to maintain long-lived T-cell memory in a stem cell-like fashion (reviewed in [20, 22]); however, it was hypothesized that a small distinct subset might have the greatest regenerative properties.

Memory T-cell maintenance: the stem cell hypothesis

A multidrug resistance-1 glycoprotein-expressing population of T_{CM} cells has been proposed to retain stem cell-like properties: similar to hematopoietic stem cells, these cells rapidly efflux chemotherapeutic drugs in vitro owing to the expression of multidrug resistance-1 [23]. These cells are preferentially found in the T_{EM} compartment, express IL-18Rα, and CD161, and have been shown to preferentially harbor the Vα7.2 chain of the TCR [24, 25]. However, $IL\text{-}18R\alpha^+CD161^{hi}$ cells do not share other properties with somatic stem cells [26] and, consistent with the overrepresentation of Vα7.2, have been shown to be mucosa-associated invariant T cells (reviewed in [27]). Given their terminally differentiated phenotype and their oligoclonality, $IL\text{-}18R\alpha^+CD161^{hi}$ cells cannot represent a cell population that is able to regenerate the memory T-cell compartment as a whole, e.g. after chemotherapy.

In vitro and in vivo studies in humans and rhesus macaques (RMs) led to the identification of a different subset of T cells with multiple stem cell-like properties, termed stem cell memory T (T_{SCM}) cells. These antigen-experienced cells comprise a relatively rare memory population having a largely T_N phenotype, being $CD45RA^+CD45R0^-CCR7^+CD62L^+CD27^+CD28^+$, while overexpressing the CD95 antigen [24, 28], expressed at high levels by all memory cells [29, 30]. T_{SCM} cells precede T_{CM} cells in differentiation, as demonstrated by their phenotype and gene expression profile. Despite having the functional capacity

of memory cells, T_{SCM} cells retain a core of genes expressed by T_N cells and share the recirculation patterns and distribution of T_N cells in vivo. T_{SCM} cells are able to self-renew in vitro: when stimulated with anti-CD3/CD28 and thus induced to differentiate, T_{SCM} cells demonstrate a twofold higher capacity to maintain the original phenotype when compared with T_{CM} cells [24]. In simian immunodeficiency virus (SIV) infected macaques, antigen-specific T_{SCM} cells have a tenfold higher capacity than T_{CM} cells to survive following the loss of the cognate antigen. Activated T_{SCM} cells were found to express higher levels of *BCL2*, *MCL1* (both antiapoptotic), and *LEF1* (regulating self-renewal) transcripts compared with T_{CM} and T_{EM} cells [28]. At the same time, T_{SCM} cells display greater multipotency, as they are capable of generating all memory subsets, including T_{CM} cells [24, 28]; no other memory subset thus far has been found to regenerate T_{SCM} cells.

Additional memory T-cell subsets

CCR7 and CD62L are mostly co-expressed on the surface of $CD4^+$ and $CD8^+$ T cells [18]. In addition, cells expressing these markers nearly uniformly express CD27 and CD28 — but the inverse is not true [31]. In particular, $CCR7^-/CD62L^-$, $CD28^+$ cells are found in the peripheral blood of healthy individuals [32, 33] or RMs [34] and constitute a subset of "transitional" memory (T_{TM}) cells. T_{TM} cells appear to be more differentiated than T_{CM} cells but not as fully differentiated as T_{EM} cells in terms of phenotype [32, 33] and magnitude of expansion in response to IL-15 in vivo [34, 35].

IL-15 administration also expands a subset of T_{EM} cells that re-express CD45RA (named terminal effector (T_{TE}) cells or sometimes T_{EMRA} cells) [35]. $CD45RA^+CCR7^-$ T_{TE} cells are found more frequently in the $CD8^+$ compartment [18], remain $CCR7^-CD62L^-$, are generally negative for CD27 and CD28, and display the shortest telomeres among T cells [36]. T_{TE} cells express markers of senescence, including KLRG-1 [37], CD57 [38], and phosphorylation of histone H2AX [39], and have low proliferative and functional capacity [38, 40], indicating terminal differentiation.

A population of noncirculating memory T cells, generally referred as tissue-resident memory T (T_{RM}) cells, is emerging as pivotal in the protection of mucosal surfaces and epithelia from invading pathogens. T_{RM} cells are identified in mice and in humans by the expression of CD69. The role of T_{RM} cells in immunity has been recently reviewed in detail elsewhere [21].

A linear model of memory T-cell differentiation based on functional properties

Functional properties are preferentially, but not uniquely, associated with given phenotypically defined subsets. Nevertheless, phenotypic, functional, and gene expression properties of these T-cell subsets [24, 28] suggest that human memory T-cell differentiation follows a linear progression along a continuum of major clusters (T_N, T_{SCM}, T_{CM}, T_{TM}, T_{EM}, and T_{TE} cells; smaller, rarely explored subsets have yet to be placed in this pathway [41]), where less differentiated cells give rise to more differentiated progeny in response to antigenic stimulation or, potentially, homeostatic signaling. With increasing differentiation, memory T cells progressively acquire or lose specific functions (Fig. 1). In mice, the precursor–progeny relationship among memory T-cell subsets can be directly addressed by physically separating cells on the basis of their phenotypic traits before adoptively transferring them into genetically identical recipient hosts. Conversely, in humans or NHPs, this relationship can only be demonstrated indirectly.

Though to some degree, the expression of "binary" markers (whose expression is either on or off) appears to be stochastic as cells progress, not all possible phenotypes (combinations of all individual markers) are found in a naturally occurring T-cell population, indicating that functional profiles are at least in part constrained by regulatory mechanisms [42].

The proposed model applies to T cells in the quiescent state. T-cell differentiation requires an obligatory activation event (i.e. TCR signaling). When activated by their cognate antigen, T cells transiently become effectors, and their phenotypes remain unpredictable by virtue of the large number of genes that are (transiently) up- or downregulated. The T-cell effector phenotype mostly coincides with that of quiescent T_{EM} cells, but effectors overexpress the activation markers CD38 and HLA-DR [43], as well as CD69 and CD25; they sometimes co-express CD45RA and CD45R0 at high levels. Following antigen clearance, activated T cells will "rest down" into any of multiple memory subsets [44], but the regulators of fate decision are still poorly understood (reviewed in [45]).

A unified phenotypic model of human T-cell differentiation

Memory T-cell differentiation defined by four phenotypic markers

According to the experimental system (i.e. mouse versus NHP versus human; steady-state versus infection) and the markers used, naïve and memory T cells have been described in various ways and nomenclatures. For instance, in mouse models of infections, the term "effector" refers to a T cell recently activated by antigen. In humans, "effector" usually designates a subset of terminally differentiated memory T cells capable of immediate inflammatory cytokine production and cytotoxicity, yet little proliferative capacity. Moreover, different markers are being used to conceptually define the same subset. A unified consensus for the terminology and phenotype of naïve and memory T-cell subsets is needed, as both semantic and experimental discrepancies are present in the community. Since multiparameter flow cytometry approaches are becoming more popular, if not routine, in immunology laboratories, we suggest that human T-cell differentiation should be

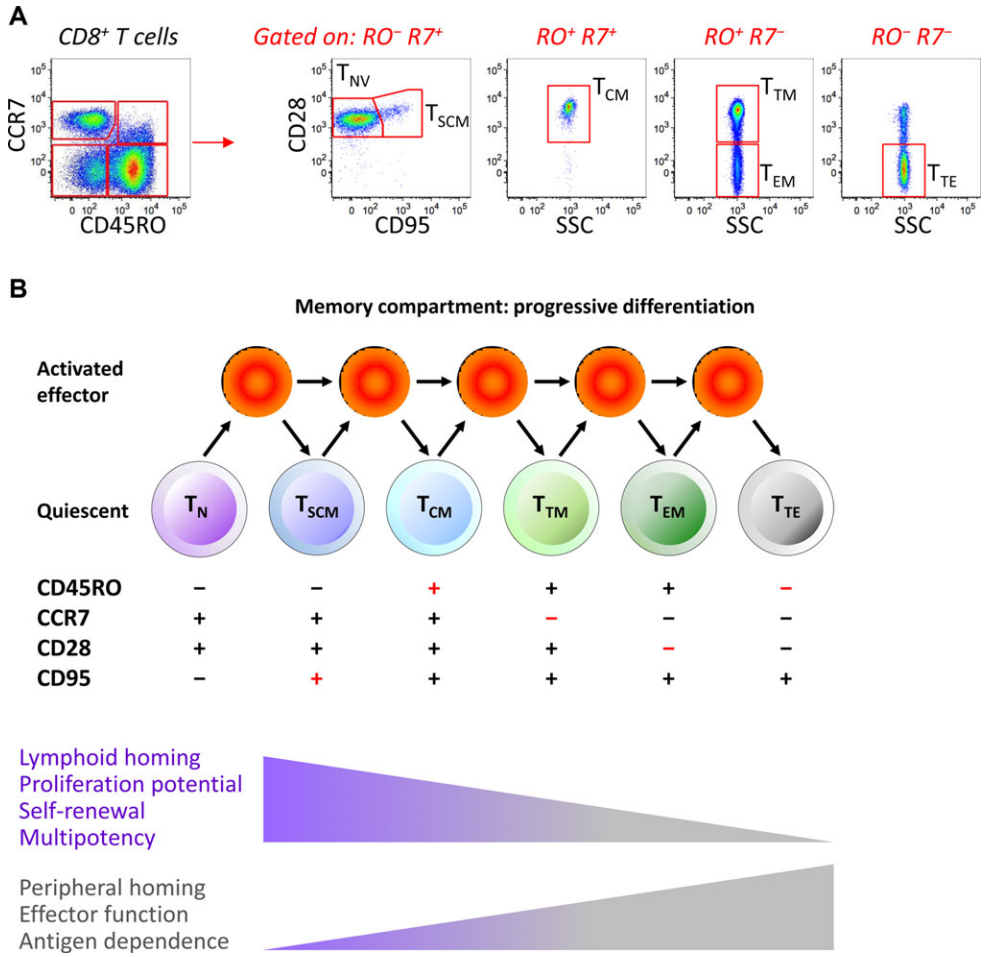

Figure 1. Heterogeneity of the memory T-cell compartment. (A) Polychromatic flow cytometry identification of multiple T_N and memory subsets in the peripheral blood according to differential expression of CD45R0, CCR7, CD28, and CD95. (B) The positive or negative expression of CD45R0, CCR7, CD28, and CD95 identifies six major subsets of quiescent T cells. Those markers that change when T cells differentiate from one subset to another are indicated in red. While differentiating (through activation) from T_{SCM} to T_{CM}, T_{TM}, T_{EM} and culminating in T_{TE} cells, memory T cells progressively lose or acquire specific functions. Following encounter with Ag, these quiescent T cells develop into effectors, which phenotype is highly dynamic and largely unpredictable. When the antigen is cleared, effector T cells that survive return to a quiescent memory state. The signals that regulate fate decision, i.e. the acquisition of a given phenotype, are largely unknown. Various cellular functions are preferentially, but not uniquely, expressed by these subsets, as shown at the bottom.

delineated using a minimum set of canonical markers, i.e. CD45R0 (or CD45RA), CCR7, CD28, and CD95. The differential expression of these markers allows the identification of six subsets in the peripheral blood of healthy humans: T_N, T_{SCM}, T_{CM}, T_{TM}, T_{EM}, and T_{TE} cells (Fig. 1). The differential expression of other molecules mediating lymphocyte functions, including migration, co-stimulation, cytotoxicity, and adhesion is shown in Table 1.

Why multiple makers are necessary

Eleven-color flow cytometric analysis of T cells has revealed that a single marker or a combination of two markers does not allow the identification of pure T_N cells [46]. Indeed, T cells expressing only two naïve markers (but not a third) are memory cells on a functional basis [46]. Ideally, the more markers are included to define T_N cells in a polychromatic panel, the better. However, some of these markers have a largely redundant expression in T_N cells, therefore not providing additional information for the exclusion of memory T-cell contaminants [47]. For instance, $CD95^+$ T_{SCM} cells have been found in a population defined by seven different T_N-cell antigens [24]. The $CD95^-$ counterparts are highly pure, and are incapable of mediating either rapid cytokine production or proliferating in response to IL-15 [24]. Moreover, clonally expanded virus-specific $CD8^+$ T cells were not detectable in $CD95^-$ T_N cells [24]. Although CD62L can replace CCR7, given their co-expression on the cell surface, CD62L expression is variably lost with freeze-thaw procedures, thus rendering its quantification unreliable in cryopreserved cells.

Given the high similarity between the human and NHP immune systems (indeed, most antihuman T-cell reagents can be used to identify T cells across many NHP species), a similar classification can be used for both, albeit with some modifications. For example, CD45RA should replace CD45R0, as antibodies

Table 1. Expression of functional molecules by circulating naïve and memory T-cell subsets according to differentiation stage[a)]

Category	Antigen	Function	Subset/species	T_N	T_{SCM}	T_{CM}	T_{TM}	T_{EM}	T_{TE}
Costimulation/survival	CD28	Costimulation		+	++	++	++	−	−
	CD27	Costimulation		++	+	+	+	−/+	−
	CD127	IL-7 signaling		++	+++	+++	++	−/+	−
	PD-1	Inhibition of effector function		−	−/+	+	++	+	+
	CD122	IL-2/IL-15 signaling		−	+	++	+++	+++	+++
	CD132	γ_c cytokine signaling		+	+	+	+	+	+
	KLRG-1	Inhibition of effector function		−	ND	−/+	+	++	+++
Activation	HLA-DR	Peptide presentation		−	−	−/+	−/+	+	−
	CD38	Calcium flux/signal transduction		+	−/+	−	−	−	−
	CD69	Proliferation		−	−	−	−	−	−
	Ki-67	Proliferation	Human	−	−	−/+	−/+	−/+	−
			Rhesus macaque	−	++	+	++	++	−
Adhesion	CD11a	Adhesion to APC/endothelium		+	++	++	+++	+++	+++
	CD58	Adhesion to APC		−	+	++	+++	+++	+++
	CD99	Transendothelial migration		−/+	+	++	++	++	++
Migration	CD62L	Secondary lymphoid tissues homing		+	+	+	−	−	−
	CD103	Gut homing		−	−	−	−	+	−
	CCR4	Chemokine response/Th2 associated		−/+	+	++	+++	+++	−/+
	CCR5	Homing to inflamed tissues		−	−	+	++	+++	++
	CCR6	Chemokine response/Th17 associated		−	−	++	+++	+++	−
	CCR9	Gut homing	$CD4^+$	−	ND	+	−	−	−
			$CD8^+$	−	ND	+	++	++	−
	CCR10	Skin homing		−	−	+	ND	++	−
	CXCR3	Homing to inflamed tissues	$CD4^+$	−	−/+	+	++	+++	+++
			$CD8^+$	++	+++	+++	++	+	+
	CXCR4	Homing to bone marrow		+	++	+++	+++	++	++
	CLA	Skin homing		−	ND	+	ND	++	ND
Cytolytic molecules	Granzyme A	Cleavage of cellular proteins	$CD4^+$	−	−	−	−	−/+	+
			$CD8^+$	−	−	−/+	++	+++	+++
	Granzyme B	Cleavage of cellular proteins	$CD4^+$	−	−	−	−	−/+	−/+
			$CD8^+$	−	−	−	+	++	+++
	Perforin	Pore forming	$CD4^+$	−	−	−	−	−/+	−/+
			$CD8^+$	−	−	−/+	+	++	+++
Miscellaneous	CD57	Unknown		−	−	−	−/+	++	+++
	CD161	Regulation of proliferation/cytotoxicity		−	−	−/+	+	+++	+++
	IL-18Ra	Response to IL-18	Human	−	−/+	+	++	+++	+++
			Rhesus macaque	−	+++	+++	+++	+++	+++
	c-Kit	Response to SCF		−	−	−	ND	+++	ND
	CD130	Response to IL-6		++	+	−/+	−	−	−

[a)] Combination of + and − indicate the expression level (either percentage or fluorescense intensity by flow cytometry) as relative to T_N cells. Differences between $CD4^+$ and $CD8^+$ T cells or between human and nonhuman primate T cells are indicated, when occurring.
ND: not determined; SCF: stem cell factor

recognizing human CD45R0 are not cross-reactive in NHPs. T_N and memory cells in NHPs have been historically defined by CD28 and CD95, where $CD28^+CD95^-$ are T_N, $CD28^+CD95^+$ are T_{CM}, and $CD28^-CD95^+$ are "effector" cells [48]. The inclusion of CD45RA and CCR7 allows the definition of T_N cells by multiple markers ($CD45RA^+CCR7^+CD28^+CD95^-$) and the identification of T_{SCM} cells ($CD45RA^+CCR7^+CD28^+CD95^+$) [28]. Therefore, defined NHP T_N cells do not respond to IL-15 in vitro or contain cells specific for the SIV [28]. NHP T_{SCM} cells are phenotypically and functionally similar to human T_{SCM} cells and display different dynamics and homeostasis in the body compared to T_{CM} cells, indicating that T_{SCM} cells constitute a discrete subset in vivo

[28]. The adoptive transfer of T_{CM}-derived effector cells demonstrated the capability of T_{CM} cells to establish persistent memory in vivo and to regenerate T_{CM}-phenotype cells, thus indicating that T_{CM} cells retain stem cell-like properties to some degree [49]. However, T_{CM} cells have been isolated as $CD28^+CD95^+$ and thus contain T_{SCM} cells as well. It would therefore be informative to perform a similar experiment on separated T_{SCM} and T_{CM} cells to determine their relative self-renewal capacity and multipotency.

CD45RA and CCR7 also allow the identification of additional memory subsets, including T_{TM} ($CD45RA^-CCR7^-CD28^+CD95^+$) [34, 35] and T_{TE} cells ($CD45RA^+CCR7^-CD28^-CD95^+$) [35, 50]. Despite the fact that the expression of CD27 and CD28 tends to be largely co-regulated in humans (although some T_{TE} cells express CD27 but lack CD28), RM T cells are uniformly positive for CD27 in the peripheral blood, independent of their differentiation stage [50] (Roederer and Lugli, unpublished observation). Hence, CD28 may be a more reliable antigen to distinguish T_{CM} and T_{TM} from T_{EM} and T_{TE} cells. The use of CD45RA as a marker of T-cell differentiation in NHPs has been questioned in the past, as CD45RA-expressing memory (at least $CD8^+$) T cells are absent from mucosal surfaces, such as lung and gut, and do not accumulate with age [50]. $CD45RA^+$ $CCR7^-$ NHP T cells express phenotypic traits of terminally differentiated memory cells, i.e. they are $CD28^-$, $CD130^-$, IL-$7R\alpha^-$, IL-$18R\alpha^+$, and $CD11a^{hi}$ [28] (Roederer and Lugli, unpublished observation). It is important to note that T_{TE} cells are relatively absent from these mucosal tissues in humans [51], suggesting that $CD45RA^+CCR7^-$ T cells represent the same subset in both humans and NHPs.

Chemokine receptor expression in human T cells

One essential feature of T cells that allow them to perform their functions is the capacity to migrate out of the blood and into different bodily tissues. Cell extravasation is controlled by a multistep process whereby adhesion molecules (selectins, chemokine receptors, and integrins) act sequentially to mediate attachment, rolling and firm adhesion of circulating T cells on endothelial cells, and finally transendothelial migration (reviewed in [52]). After traversing the endothelium and the subendothelial basement membrane, T cells migrate through the interstitial tissues and localize to different microenvironments following gradients of chemokines produced by tissue resident or infiltrating cells. The specificity of this process is determined by differential expression of adhesion molecules on T cells and their ligands on endothelial cells and tissues [53]. For instance, T_N and T_{CM} cells express CD62L, CCR7, and ICAM-1, which are required to adhere to and cross the high endothelial venules of LNs and to migrate to the DC-rich T-cell zone. Upon interaction with Ag-presenting DCs, activated T cells upregulate CXCR5 [54] and move toward the T-cell/B-cell border to interact with Ag-activated B cells and differentiate into follicular helper T (T_{FH}) cells. Human $CXCR5^+$ T_{FH} cells are found in the B-cell follicles and express costimulatory molecules that are required to provide help to B cells [55, 56], thus driving the development of germinal centers and the establishment of serological memory (reviewed in [57]).

Chemokine receptor expression in effector T-cell specification

Chemokine receptors have been particularly useful for dissecting human T-cell subsets with distinct migratory capacity and effector function. The expression of chemokine receptors is not only the basis for a distinction between T_{CM} and T_{EM} cells, but it can be also used to identify and isolate T cells that produce distinct cytokines or display different activation states or tissue tropisms. This is the case for CXCR3, expressed on IFN-γ-producing type 1 helper cells (T_H1); CCR4, expressed on IL-4-producing T_H2 cells; and CCR6, expressed on IL-17-producing T_H17 cells (reviewed in [58,59]). CCR5 is preferentially expressed on activated (and T_{EM}) T_H1 cells, while CRTh2, the prostaglandin D2 receptor, is expressed on a small subset of lineage-committed T_H2 cells [60, 61]. CCR9, together with $\alpha4\beta7$, marks T cells that can migrate to the intestine, while CCR4, CCR10, and CLA are expressed on skin-homing T cells (reviewed in [62]). Most skin-homing T cells produce IL-22 and may represent a distinct subset of T_H cells (termed T_H22) [63, 64]. T_H22 cells have been shown to stimulate keratinocytes to produce antimicrobial peptides and are found in certain types of inflammatory skin diseases, such as psoriasis [65].

While there is clear evidence that the regulation of homing receptor expression is an integral part of the T-cell differentiation program, the exact mechanisms and the precision of this regulation have not been fully elucidated. It is possible that the same transcription factors that induce cytokine gene expression in differentiating T cells also regulate lineage-related chemokine receptors. This possibility is supported by the demonstration that T-bet regulates the expression of IFN-γ and CXCR3 in T_H1 cells [66]. Similarly, RORγt regulates IL-17 and CCR6 expression in developing T_H17 cells [67, 68].

Using chemokine receptors as surface markers for cell sorting, one can study the antigenic repertoire of functionally distinct T-cell subsets. These studies provide relevant insights into the complexity of the human immune response to pathogens, as well as the compartmentalization of the response to self-Ags or allergens [67, 69–72]. For instance, the measurement of four chemokine receptors allows the isolation of five memory T_H subsets from the PBMCs of healthy donors: (i) $CXCR3^+CCR6^-$ T_H1 cells; (ii) $CXCR3^+CCR6^+$ T_H17/T_H1; (iii) $CCR4^+CCR6^-$ T_H2; (iv) $CCR6^+CCR4^+$ T_H17; and (v) $CCR4^+CCR10^+$ T_H22. The analysis of T-cell specificities present in these subsets has revealed that influenza-specific memory T_H cells are found in the T_H1 and T_H17/T_H1 subsets, while cells specific for *Mycobacterium tuberculosis* are almost exclusively present in the $CCR6^+$ T_H17/T_H1 subset [69]. Memory T cells specific for *Streptococcus pyogenes* are found primarily in cells derived from the $CCR6^+$ subsets (T_H17 and T_H17/T_H1), while memory T cells specific for *Candida albicans* are most prominent in the T_H17 subset [67], but reactive

cells are also detected in CCR6$^+$ T_H17/T_H1, CCR4$^+$ T_H2, and CCR10$^+$ T_H22 cells (reviewed in [73]). This approach, which is extremely powerful when combined with high-throughput cellular screening methods [74], will continue to provide insight into the human immune response in infections, vaccinations, and diseases.

It is important to note that TCR stimulation induces a transient switch in chemokine receptor expression in already differentiated memory T cells (consistent with the dynamic and often unpredictable phenotype of activated cells). For instance, CCR6 and CXCR3 are downregulated in activated T cells, while CCR4 is rapidly upregulated [18]. CCR7 and CXCR5 are also transiently upregulated in all memory T-cell types [19]. Thus, Ag stimulation of T_{EM} cells that have migrated to peripheral tissues may lead to induction of CCR7 and CXCR5, needed to enter afferent lymphatic vessels and circulate back to the draining LNs and the B-cell follicles.

Chemokine receptor expression in memory T-cell differentiation

Considering that there are 19 well-defined chemokine receptors for which monoclonal antibodies are available and that T cells express multiple chemokine receptors, there are seemingly endless possibilities for combinations of expression and therefore tremendous phenotypic and possibly functional diversity. A combination of nine antibodies to chemokine receptors (CCR7, CCR4, CXCR3, CCR5, CXCR5, CCR3, CRTh2, CCR6, and CCR10) has revealed that naïve T cells, defined as CD45RA$^+$CCR7$^+$CD95$^-$, homogeneously express CCR7 — but a few also express CXCR3, CXCR5, CCR4, or CCR6 [75]. The basis for this phenotypic heterogeneity in a subset that is considered functionally homogeneous is unclear at the moment. It is interesting to note that the same receptors are expressed at higher levels on CD45RA$^+$CCR7$^+$CD95$^+$ T_{SCM} cells. Some receptors, such as CCR10, CCR5, CRTh2, and CCR6, are increasingly expressed as cells become more differentiated, progressing from T_{SCM} to T_{CM} and T_{EM}, with the exception of the terminally differentiated T_{TE} population that is lacking all receptors, except CCR5. CXCR3 is highly expressed in both T_{CM} and T_{EM} subsets, and lacking in T_{TE} cells, while CXCR5 and CCR9 are expressed more in T_{CM} cells and less in T_{EM} cells. These dynamics of chemokine receptor expression from T_N, to T_{SCM}, to T_{CM}, to T_{EM} until complete exhaustion and loss of most receptors at the T_{TE} stage, is consistent with the dynamics of gene expression revealed by genome-wide studies [24, 76] and further support the linear T-cell differentiation model.

Coordinated cytokine expression by human T-cell subsets

As T_N cells are activated by encounter with cognate Ag they are induced to differentiate, proliferating as they do so and acquiring effector functions, namely the production of cytokines as well as cytolytic molecules. These molecules are required to shape the inflammatory milieu of an immune response, drive the expansion and identity of immune cells, and kill infected or malignant cells. The expression pattern of such effector molecules varies between individual differentiation stages [6]. Similarly, different cytokines and combinations thereof are induced by specific Ags, such as CMV, EBV, and influenza virus, resulting in prevalent functional and phenotypic signatures of the corresponding Ag-specific T cells [42, 77]. CD4$^+$ and CD8$^+$ T cells producing only one versus two or three cytokines have varying differentiation signatures (Fig. 2).

Effector cytokines: IL-2, IFN-γ, and TNF

IL-2 is typically considered an early cytokine, being induced shortly after T-cell receptor (TCR) triggering, particularly in CD4$^+$ T cells. It is typically found in less differentiated cells such as T_{SCM} and T_{CM} cells, especially when produced in the absence of either TNF or IFN-γ, and is important in determining proliferation, differentiation, and survival of Ag-specific T cells. In fact, while IL-2 drives T-cell differentiation, strong and persistent IL-2 exposure progressively promotes short-lived CD8$^+$ effector cells at the cost of inducing cells with memory capacity [78, 79].

TNF is also one of the earliest effector molecules produced by activated T cells. Aside from mediating lysis against certain tumors, it is a potent pyrogen that enhances fluid drainage and cellular motility across endothelia by activating the vascular endothelium, which is important in recruiting immune cells such as polymorphonuclear cells to the site of inflammation [80–82]. The ability to produce TNF is lost in later differentiation stages such as in T_{TE} cells, most dramatically in the CD4$^+$ compartment [77].

In contrast to IL-2 and TNF, efficient IFN-γ production requires several rounds of proliferation [83] and thus occurs mostly in T cells at later stages of differentiation [17, 32, 84]. Nonlymphoid tissue resident T_{EM} and T_{TE} cells demonstrate more robust IFN-γ production than T_{CM} cells, providing a mechanism to rapidly respond to re-infections. IFN-γ activates macrophages, induces elevated expression of MHC class I and II molecules on T cells, directly inhibits viral replication, and stimulates the cytolytic activity of NK cells; thus it is a key player in orchestrating immune responses to invading organisms and malignancies.

Cytolytic molecules: Granzyme A, granzyme B, and perforin

As with cytokines, there is a hierarchical expression of cytolytic molecules in memory T-cell subsets. Among granzymes (Gr) A and B and perforin, GrA is the first to become detectable during differentiation into memory cells, followed by GrB and later perforin [36, 85, 86]. In fact, GrB is never expressed in the absence of GrA. Likewise, perforin-positive cells are primarily positive for GrA and GrB, making it a choice indicator for dedicated cytolytic cells [86]. Interestingly, perforin expression shows an inverse correlation with cell surface expression of CD27 [87] and CD28, as

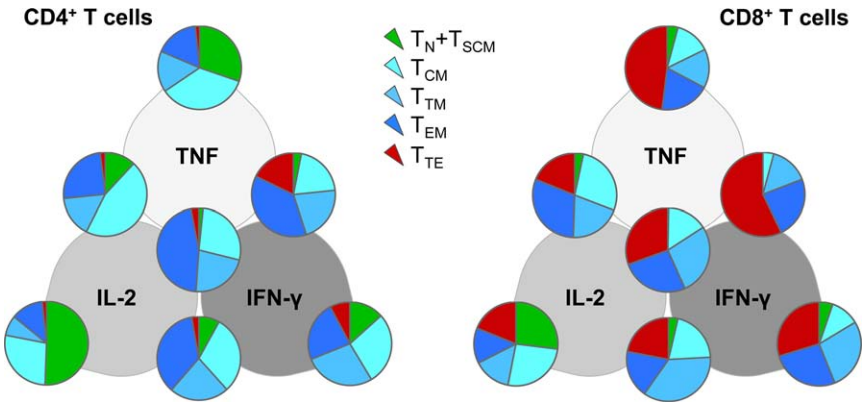

Figure 2. Different signatures of T cells with defined cytokine potential. Healthy donor $CD4^+$ and $CD8^+$ T cells stimulated with staphylococcal enterotoxin B can produce diverse combinations of IFN-γ, IL-2, or TNF (i.e. a single cytokine alone, all three cytokines simultaneously, or various combinations of two cytokines). The location of the pies (intersecting the IFN-γ, IL-2, or TNF circles) indicates the cytokines produced, while pie patterns represent the diversity of differentiation phenotypes (represented by color-coded sectors in the pie that are defined by surface expression of CD45RO, CCR7, and CD28; larger sectors indicate higher proportions of a certain phenotype) of cellular populations producing the given cytokine combination. This representation highlights that some cytokine combinations are more prone to be produced by less differentiated cells, while others are mostly found in more differentiated cells. While $CD4^+$ T cells producing one cytokine only (pie charts in the outside corners, intersecting one circle only), in particular IL-2 or TNF, have a predominantly early differentiated phenotype (T_N/T_{SCM} and T_{CM} populations), cells producing two (pie charts straddling two cytokine areas) or three cytokines (center pie chart) are more enriched in T_{EM} and T_{TE} cells. $CD8^+$ T cells generally need to undergo more significant differentiation (higher proportion of T_{TM}, T_{EM}, and T_{TE} cell sectors in the pies) than $CD4^+$ T cells before producing cytokines, with the most prevalent cytokine signature of T_N/T_{SCM} cells being IL-2 in the absence of either IFN-γ or TNF.

well as a positive correlation with the senescence marker CD57, which can be used as a surrogate marker for T cells with high cytolytic potential [86].

Cytotoxicity was thought to be limited to $CD8^+$ T cells (hence they were named "cytotoxic T lymphocytes" or "CTLs"), while $CD4^+$ T cells were considered to be mostly cytokine-producing cells (hence named helper or T_H cells). It is now recognized that $CD4^+$ T cells also express granzymes [88] and perforin [89] as well as other cytolytic molecules [90], kill target cells [91], degranulate in response to peptide stimulation [91, 92] and, through their cytotoxic behaviors, may influence the course of HIV infection [93]. In a similar way, human $CD8^+$ T cells have recently been shown to produce CD154 (CD40 ligand or CD40L) upon antigen-specific stimulation and to provide help to B cells and DCs [94,95]. The role of these cells in protection from pathogenic infections still has to be elucidated.

Turnover and in vivo persistence of T-cell subsets

T cells in the circulation of human healthy donors are largely quiescent, although a small proportion in the peripheral blood (generally less than 1%) is actively cycling, as revealed by Ki-67 expression on these cells [96]. Ki-67 is a nuclear antigen that is expressed by proliferating cells, irrespective of the cell cycle phase [97]. Ex vivo, the majority of $Ki\text{-}67^+$ cells harbor a T_{EM} phenotype [24,96]. Ki-67 expression is generally accompanied by the activation markers HLA-DR and CD38. Conversely, T_N cells do not express Ki-67, suggesting that individual T-cell subsets differ in their in vivo turnover rate [24]. RM T_{SCM} cells harbor the highest Ki-67 expression among memory subsets in the circulation but not in peripheral tissues [28], and display a rate of bromodeoxyuridine (BrdU) incorporation similar to that of T_{EM} cells and higher than that of T_{CM} cells following acute SIV infection, indicating a rapid turnover despite their "young" phenotype [28]. This could be, at least in part, explained by the generally more elevated immune activation of NHPs compared with that of human T cells [98].

In vivo turnover of human T-cell subsets

T-cell phenotypes are unstable following activation and tend to acquire characteristics of effectors irrespective of the original population (Fig. 1). Therefore, the relative turnover of different T-cell subsets cannot be determined solely by the expression of Ki-67 and should thus be integrated with other, more sophisticated measurements. Human T-cell turnover in vivo could also be measured by stable isotope labeling with deuterated glucose or deuterated water. These molecules are metabolized by the cell and are stably incorporated into the DNA upon division. While glucose is generally infused over a period of 24 h and preferentially incorporated by cells with a rapid turnover, water is administered for a long period of time, and labels slow-dividing cells [99]. Following deuterated glucose administration, circulating $CD4^+CD45RA^+CCR7^+$ T_N cells are uniformly (>99%) nondividing. Conversely, human memory cells have been found to incorporate more deuterium, with T_{EM} cells incorporating the highest levels of deuterium [100]. While deuterium levels in T_{CM} cells tend to remain constant over the observation period of 25 days, with some variability among subjects, those of T_{EM} and T_{TE} cells rapidly decay over time, indicating that these subsets undergo turnover in vivo. From these observations, Macallan et al. [100] estimated that a T_N cell doubles on an average about

once a year, a T_{CM} cell every 60 days, and T_{EM} or T_{TE} cell every 20 days [100]. In a different study, however, healthy subjects were given deuterated water over a period of 9 weeks, to achieve the long-term labeling of slowly dividing T cells [101]. In this study the T_N-cell half-life was estimated to be between 1517 and 2374 days while that of memory T cells was estimated at 155 to 244 days [101]. In these studies, T_N cells were defined on the basis of two markers only, and therefore contained memory T-cell contaminants, including the T_{SCM} cells. It is thus likely that the T_N-cell compartment is even longer-lived than has been determined experimentally.

Despite being informative on the relative turnover of naïve and memory cells in vivo, the two studies [100,101] provided very different results, due to the differences in the reagents used for labeling, the administration protocols and the markers used to identify the T cells. The duration of the labeling period has been recently proposed as a major cause of the discrepancies of in vivo labeling studies [102]. Moreover, deuterated glucose and water require cell division to be incorporated into the DNA, thus limiting the analysis to dividing cells. It is not known whether cells belonging to a given T-cell subset are potentially heterogenous in turnover.

Loss of survival capacity with progressive memory differentiation

In these studies, the half-life of a given population was considered to be influenced by cell death, trafficking between tissues and disappearance due to phenotypic changes (differentiation) [99]. The biological implication, therefore, is that early differentiated memory T cells are longer-lived in vivo compared to more terminally differentiated T cells. Over the years, this has been supported by a number of in vivo and in vitro experiments (reviewed in [103]). T_{CM} cells, the prototype of early-differentiated memory cells prior to the identification of T_{SCM}, display increased proliferative capacity, self-renewal, and multipotency compared to T_{EM} cells. Moreover, T_{CM} cells retain a gene expression signature associated with protection from apoptosis, i.e. increased levels of IL-7R [104] or STAT5a and lower levels of the proapoptotic protein Bim [105]. $CD4^+$ T_{CM} cells have a lower tendency to undergo both spontaneous and CD95-induced apoptosis in vitro compared with $CD4^+$ T_{EM} cells [105], as observed among HIV- and CMV-specific $CD8^+$ T cells in HIV-infected patients [106]. According to the assumption that a hierarchy exists among the memory T-cell compartment, T_{SCM} cells display superior survival capacity following the loss of the cognate antigen in acute SIV infection, are less apoptotic in vitro and preferentially express genes associated with long-term maintenance (LEF1) and survival (BCL2 and MCL1) compared with conventional memory subsets [28].

In vivo correlates of persistence following adoptive T-cell transfer

Adoptive T-cell transfer based clinical trials suggest that the capability to persist in vivo is dependent on the differentiation stage. The cells employed in these trials display terminal differentiation and, following transfer, fail to persist in the long term in vivo [107]. Conversely, effector cells expanded in vitro from early differentiated cells, such as from the T_{CM} cells [49], or bearing features associated with the T_{CM} stage, correlate with improved persistence [103].

Although these and other trials identified correlates of persistence in vivo, it was not possible to determine the relative survival and expansion capacity of discrete T-cell subsets, given the heterogeneity of the initial population. When sorted to high purity by multicolor fluorescence-activated cell sorting and transferred into highly immunodeficient NOD/SCID/IL-2Rγ $chain^{-/-}$ mice, naturally occurring human T_{SCM} cells demonstrate 10- to 100-fold more potent proliferative capacity in comparison to T_{CM} or T_N cells [24]. When generated from T_N precursors by either inhibiting the GSK-3β pathway [24] or by co-stimulating with IL-7 and IL-15 [108], the induced T_{SCM} cells mediate superior antitumor capacity in a humanized mouse model of mesothelioma and are able to perpetuate graft versus host disease upon serial transplantation experiments, respectively. T_{SCM} cells are, therefore, not only capable of persisting (owing to their stem cell-like properties) but also of differentiating into potent effectors in response to antigenic stimulation. Conversely, T_{EM}-derived effector cells, which are generally employed in adoptive T-cell transfer immunotherapy trials, fail to engraft and/or persist, thus indicating that they lack stem cell-like properties [24,49,108]. Only dedicated preclinical/clinical trials in NHPs or patients using purified T-cell subsets will unequivocally demonstrate the self-renewing and multipotent capacity of T cells at different stages of differentiation.

Concluding remarks

High-content analysis of protein expression at the single-cell level has revealed that the memory T-cell compartment is a lot more heterogenous than previously thought. Indeed, it is necessary to overcome the T_{CM}- and T_{EM}-cell dichothomy and integrate the models of human memory T-cell differentiation with the newly discovered cell subsets with potential implications in disease or T-cell-based therapeutic approaches. The use of different markers for the identification of a given subset is, at least in part, the basis of the discrepancies in the results obtained from different laboratories. As mentioned previously, studies on naïve cells isolated solely by the use of one or two markers would be influenced by the contamination of memory cells (up to 20%). The detailed classification proposed here is necessary to identify pure subsets with distinct properties. As the field continues to evolve, it is becoming apparent that single cells with a given phenotype are not created equal, as they can differ in gene expression [109] or in vivo function [110,111]. Future in depth analysis of immune functions at the level of single cell will lead to the identification of master regulators of T-cell differentiation. With such information in hands, we will be able to modulate human T-cell fate decisions by pharmacological interventions and thus promote stem cell-like

qualities or rapid effector functions to be utilized in vaccination and adoptive cell transfer immunotherapies.

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Abbreviations: **CCR7**: C-C chemokine receptor 7 · **NHP**: nonhuman primate · **RM**: rhesus macaque · T_{CM}: central memory T cell · T_{EM}: effector memory T cell · T_N: naïve T cell · T_{SCM}: stem cell memory T cell · T_{TE}: terminal effector T cell · T_{TM}: transitional memory T cell

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