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Authors
Rosenblum, Michael D
Way, Sing Sing
Abbas, Abul K

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Regulatory T cell memory

Michael D. Rosenblum[1], Sing Sing Way[2] and Abul K. Abbas[3]

Abstract | Memory for antigen is a defining feature of adaptive immunity. Antigen-specific lymphocyte populations show an increase in number and function after antigen encounter and more rapidly re-expand upon subsequent antigen exposure. Studies of immune memory have primarily focused on effector B cells and T cells with microbial specificity, using prime–challenge models of infection. However, recent work has also identified persistently expanded populations of antigen-specific regulatory T cells that protect against aberrant immune responses. In this Review, we consider the parallels between memory effector T cells and memory regulatory T cells, along with the functional implications of regulatory memory in autoimmunity, antimicrobial host defence and maternal–fetal tolerance. In addition, we discuss emerging evidence for regulatory T cell memory in humans and key unanswered questions in this rapidly evolving field.

In addition to the ability to respond to an almost infinite range of foreign antigens, the cells of the adaptive immune system can also 'remember' prior antigen encounters. Despite a fairly rudimentary knowledge of the mediators responsible for immunological memory, Edward Jenner first recognized this remarkable facet of adaptive immunity more than 200 years ago through experimental cowpox vaccination. More recently, we have come to understand that immunological memory is conferred by specialized adaptive immune cells that robustly expand upon primary antigen exposure and that retain the ability to respond with more accelerated kinetics upon subsequent encounter with the same antigen. To exploit immune memory against microorganisms, vaccines are now being engineered to induce long-term persistence of protective pathogen-specific antibodies, along with antibody-producing B cells and effector T cells. However, these findings also raise exciting new questions about whether newly identified regulatory immune cell subsets can also remember previous antigenic exposures.

Memory T cells have an essential role in immunity against microbial pathogens. As our appreciation of the diversity of functional T cell lineages has increased, so has our recognition of the memory features that are shared among many T cell subsets. Immunological memory has been most extensively characterized for $CD8^+$ T cells. Long-standing work in this field has established the existence of multiple subsets of memory $CD8^+$ T cells, which differ in terms of their tissue distribution and their capacity to traffic between peripheral tissues and lymphoid organs. These memory $CD8^+$ T cell subsets can be distinguished on the basis of their expression of cell surface markers and transcription factors, along with their distinct epigenetic landscapes and metabolic profiles (reviewed in REFS 1–3) (TABLES 1,2).

Compared with $CD8^+$ T cells, memory within the $CD4^+$ T cell compartment is less well understood. This probably stems from reduced proliferation kinetics and expansion potential that make enumerating $CD4^+$ T cells with defined antigen-specificity technically more difficult. $CD4^+$ T cells differentiate into functionally distinct effector subsets, including T helper 1 (T_H1), T_H2, T_H17 and T follicular helper (T_{FH}) cell subsets, each of which is responsible for activating specialized immunological pathways for optimal host defence against a range of microbial pathogens. This diversity makes it more challenging to quantify antigen-specific $CD4^+$ memory T cells. In addition, each effector $CD4^+$ T cell subset has inherent plasticity that further complicates tracking the persistence of functional memory $CD4^+$ T cells. Another interesting distinction between memory $CD4^+$ T cells compared with $CD8^+$ T cells relates to durability. Although $CD8^+$ T cells have consistently been shown to be maintained as a stable memory pool for extended time periods, antigen-specific memory $CD4^+$ T cells decline in number over time[4–6]. Nevertheless, antigen-specific $CD4^+$ T cells from each effector subset have been shown to persist long term after antigen elimination, as determined by unique expression patterns of transcription factors, cytokines, adhesion molecules and chemokine receptors[7,8] (TABLES 1,2).

In contrast to effector $CD4^+$ T cell subsets that promote pro-inflammatory responses, the forkhead box P3 (FOXP3)-expressing regulatory T (T_{Reg}) cell subset has potent immune suppressive properties[9,10]. Conceptually, the need for immunological memory within the effector T cell compartment is obvious — the ability to remember

[1]*Department of Dermatology, University of California San Francisco, San Francisco, California 94143, USA.*
[2]*Division of Infectious Diseases and Perinatal Institute, Cincinnati Children's Hospital, Cincinnati, Ohio 45229, USA.*
[3]*Department of Pathology, University of California San Francisco, San Francisco, California 94143, USA.*
Correspondence to A.K.A.
Abul.Abbas@ucsf.edu

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Table 1 | **Markers for memory T cell subsets***

Memory T cell subset		Mouse phenotype	Human phenotype
Conventional T cells	Central memory	CCR7^{hi}, CD44^{hi}, CD127^{hi}, L-selectin^{hi} and KLRG1^{low}	CD44^{hi}, CD45RO^{hi}, CD45RA^{low}, CD127^{hi} and express high levels of IL-2 and intermediate levels of IFNγ and TNF
	Effector memory	CCR7^{low}, CD44^{hi}, CD127^{hi}, L-selectin^{low} and KLRG1^{hi}	CD44^{hi}, CD45RO^{hi}, CD45RA^{low}, CD127^{hi}, L-selectin^{low}, express high levels of IFNγ and TNF and express low levels of IL-2
	Tissue-resident memory	CCR7^{low}, CD69^{hi}, CD103^{hi} and KLRG1^{low}	CD45RO^{hi}, CD45RA^{low}, CD69^{hi}; and CD103^{hi} and CD103^{low} subsets
	Stem cell memory	CD44^{low}, L-selectin^{hi}, CD95^{hi}, CD122^{hi}, SCA1^{hi}, BCL-2^{hi} and CD127^{hi}	CD27^{hi}, CD28^{hi}, CD45RO^{low}, CD45RA^{hi}, CD95^{hi}, CD122^{hi}, CD127^{hi}, L-selectin^{hi}, CCR7^{hi} and express low levels of IFNγ and intermediate levels of IL-2
Memory regulatory T cells‡		CD25^{hi}, CD27^{hi}, CD44^{hi}, FOXP3^{hi}, L-selectin^{low}, CTLA4^{hi} and CD127^{hi}§	CD25^{hi}, CD27^{hi}, CD44^{hi}, CD45RO^{hi}, CD45RA^{low}, CCR7^{low}, FOXP3^{hi}, L-selectin^{low}, CTLA4^{hi}, CD127^{low}, ICOS^{hi}, BCL-2^{hi}, Ki67^{low} and HLA-DR expression not defined

BCL-2, B cell lymphoma 2; CCR7, CC-chemokine receptor 7; CTLA4, cytotoxic T lymphocyte antigen 4; FOXP3, forkhead box P3; ICOS, inducible T cell co-stimulator; IFNγ, interferon-γ; IL-2, interleukin-2; KLRG1, killer cell lectin-like receptor subfamily G member 1; SCA1, stem cell antigen 1; TNF, tumour necrosis factor. *Table compiled from REFS 1,7,11–16,19,29,30,40,64,65,74,103–106. ‡It is currently unknown whether memory regulatory T cell subsets exist in mice or humans. §Shown only in mouse skin.

and to robustly respond to eradicate pathogenic microorganisms more efficiently after secondary infection would enhance survival by augmenting immunity against recurrent infection. By contrast, the biological benefit of T_{Reg} cell memory is less apparent. It has been postulated that memory T_{Reg} cells mitigate tissue damage during the heightened responses of pro-inflammatory memory cells. In addition, memory T_{Reg} cells promote reproductive fitness by reinforcing fetal tolerance during pregnancy. The importance of regulatory memory is supported by several recent studies that identify long-term persistence of antigen-specific T_{Reg} cells with potent immunosuppressive properties despite the elimination of cognate antigen[11–15]. In this Review, we describe accumulating evidence for the existence of memory T_{Reg} cells and discuss the properties and the physiological functions of this newly identified cell population.

Regulatory memory

The concept and definition of memory T_{Reg} cells. During a primary immune response, antigen-presenting cells (APCs) activate T cells by presenting antigen and by providing additional co-stimulatory signals. This results in the expansion and functional differentiation of the T cells. Effector T cells that are generated migrate to the site of infection and eliminate the offending organism. Effector T cells are fairly short-lived cells; their life cycle is defined by a rapid expansion phase, a functional inflammatory or cytotoxic phase and a contraction phase during which they undergo apoptotic cell death. These cells are mainly present during active microbial infections. By contrast, during a primary response, a subset of T cells is generated that has potential for long-term survival — this subset is termed memory T cells. Memory T cells escape apoptosis during the contraction phase and persist in either secondary lymphoid organs — in the case of central memory T cells (T_{CM} cells) — or in the recently infected peripheral tissue — in the case of effector memory T cells (T_{EM} cells) and tissue-resident memory T cells[16] (T_{RM} cells) — for prolonged periods of time after their cognate antigens have been cleared (FIG. 1). Upon re-exposure to antigen (that is, during the secondary immune response), memory T cells undergo rapid population expansion and mediate more robust effector functions compared with the primary immune response, which leads to rapid clearance of the infection. On the basis of this fundamental understanding of primary and secondary immune responses, several criteria have been suggested to distinguish memory T cells from effector T cells. It is generally accepted that essential features of memory T cells include first, evidence of prior expansion and/or activation, second, persistence in the absence of cognate antigen and third, enhanced functional activity upon antigen re-exposure.

Defining memory T cells by phenotypic markers. Classical definitions of immunological memory are based on our understanding of memory effector T cells. In the CD4$^+$ T cell lineage, evidence of prior activation includes increased expression of CD44 and reduced expression of L-selectin (also known as CD62L), which enables migration to peripheral tissues by decreasing adhesion to high endothelial venules in secondary lymphoid organs[17,18]. As interleukin-7 (IL-7) signalling promotes long-term survival of T cells, expression of high levels of CD127 (also known as IL-7 receptor subunit-α) has been used as an additional marker of effector T cell memory[19,20]. In addition, CD47, the transcription factor T-bet, LY6G and specific epigenetic landscapes have all been used as evidence of prior activation and/or differentiation in mouse memory effector CD4$^+$ T cells[21–24].

Although a growing number of markers that reliably identify memory effector T cells have been identified (TABLES 1,2), similar indicators of functional memory for T_{Reg} cells are less clearly defined. This has been complicated by the fact that many of the markers used to

Memory T_{Reg} cells
Previously activated regulatory T (T_{Reg}) cells that persist in the absence of antigen expression or in the presence of intermittent low-level antigen expression. It is currently unknown whether central memory T cell, effector memory T cell or tissue-resident memory T cell subsets of memory T_{Reg} cells exist.

Central memory T cells
(T_{CM} cells). Generated in secondary lymphoid tissues and reside in secondary lymphoid tissues in the absence of antigen.

Effector memory T cells
(T_{EM} cells). Generated in secondary lymphoid tissues and recirculate between blood and non-lymphoid tissues in the absence of antigen.

Tissue-resident memory T cells
(T_{RM} cells). Generated in non-lymphoid tissues and stably reside in these tissues in the absence of antigen.

Table 2 | **Selected markers for resting, effector and memory T cell subsets***

	Conventional T cells			Regulatory T cells		
	Resting	**Activated effector**	**Memory**	**Resting**	**Activated effector**	**Memory**
Selected phenotypic markers	$CD25^{low}$ $CD44^{low}$ $CD45RA^{hi‡}$ $CD45RO^{low‡}$ $CD69^{low}$ $L\text{-selectin}^{hi}$ $CD127^{high}$ $Ki67^{low}$ $BCL\text{-}2^{hi}$	$CD25^{hi}$ $CD44^{hi}$ CD45RA expression variable‡ CD45RO expression variable‡ $CD69^{hi}$ $L\text{-selectin}^{low}$ $CD127^{low}$ $Ki67^{hi}$ $BCL\text{-}2^{low}$ $KLRG1^{hi}$	$CD25^{low}$ $CD44^{hi}$ $CD45RA^{low‡}$ $CD45RO^{hi‡}$ CD69 expression variable L-selectin expression variable $CD127^{hi}$ $CD27^{hi}$ $Ki67^{low}$ $BCL\text{-}2^{hi}$	$CD25^{hi}$ $CD44^{hi}$ $CD45RA^{hi‡}$ $CD45RO^{low‡}$ $CD69^{low}$ $L\text{-selectin}^{hi}$ $CD127^{low}$ $CTLA4^{low}$ $ICOS^{low}$ $HLA\text{-}DR^{low‡}$ $Ki67^{low}$ $BCL\text{-}2^{hi}$	CD25 expression variable $CD44^{hi}$ $CD45RA^{low‡}$ $CD45RO^{hi‡}$ $CD69^{hi}$ $L\text{-selectin}^{low}$ $CD127^{low}$ $CTLA4^{hi}$ $ICOS^{hi}$ $HLA\text{-}DR^{hi‡}$ $Ki67^{hi}$ $BCL\text{-}2^{low}$ $KLRG1^{hi}$	$CD25^{hi}$ $CD44^{hi}$ $CD45RA^{low‡}$ $CD45RO^{hi‡}$ CD69 expression unknown $L\text{-selectin}^{low}$ $CD127^{hi§}$ $CTLA\text{-}4^{hi}$ $ICOS^{hi}$ HLA-DR expression not defined $CD27^{hi}$ $Ki67^{low}$ $BCL\text{-}2^{hi}$ KLRG1 expression not defined
Chemokine receptors	$CCR7^{hi}$	Several, including CCR3, CCR6, CCR8 and CXCR3	Variable levels of CCR7	$CCR7^{hi}$	$CCR7^{low}$	$CCR7^{low}$
Transcription factors	$FOXP3^{low}$ KLF2	FOXP3 expression variable‡ Several, including T-bet (T_H1 cell-associated), GATA3 (T_H2 cell-associated), RORγ (T_H17 cell-associated) and BCL-6 (T_{FH} cell-associated)‖	$FOXP3^{low}$	$FOXP3^{hi}$	$FOXP3^{high}$ Several others, including T-bet and IRF4	$FOXP3^{hi}$

BCL, B cell lymphoma; CCR CC-chemokine receptor; CTLA4, cytotoxic T lymphocyte antigen 4; CXCR, CXC-chemokine receptor; FOXP3, forkhead box P3; GATA3, GATA-binding factor 3; ICOS, inducible T cell co-stimulator; IRF4, IFN-regulatory factor 4; KLF2, Krueppel-like factor 2; KLRG1, killer cell lectin-like receptor subfamily G member 1; RORγ, retinoic acid receptor-related orphan receptor-γ; T_{FH}, T follicular helper; T_H, T helper. *Table compiled from REFS 1,7,11–16,19,29, 30,40,64,65,74,103–106. ‡Human only. §Shown only in mouse skin. ‖$CD4^+$ T cells only.

identify memory effector T cells cannot be applied to T_{Reg} cells. Almost all T_{Reg} cells in secondary lymphoid organs and peripheral tissues express high levels of CD44 (REFS 25,26), which makes CD44 expression of little use in defining prior activation of T_{Reg} cells. In fact, there are few T_{Reg} cell-intrinsic molecules linked with immune suppression that have been shown to be expressed *de novo* on T_{Reg} cells upon activation. Instead, activated T_{Reg} cells generally increase their expression of molecules that they already express in the steady state. For example, upon encounter with antigen, T_{Reg} cells increase their expression of cytotoxic T lymphocyte antigen 4 (CTLA4), CD25 (also knwn as IL-2 receptor subunit-α), inducible T cell co-stimulator (ICOS), and glucocorticoid-induced TNFR-related protein (GITR; also known as TNFRSF18), all of which are expressed at fairly high levels on resting T_{Reg} cells[27,28]. Quantitative shifts in expression of these proteins are therefore the most useful markers of prior antigen encounter but are not definitive. Despite these caveats, some markers that are used to define T_{EM} cells may be of value in defining memory T_{Reg} cells. It has been shown that a population of memory T_{Reg} cells in mouse skin can express high levels of CD127, which is expressed at low levels on T_{Reg} cells in secondary lymphoid organs[29]. However, this does not seem to be true for T_{Reg} cells with a memory phenotype found in human skin[30], which suggests that this marker is not a robust indicator of memory T_{Reg} cells across species. In addition, expression of CD127 will probably vary with respect to the specific location at which memory T_{Reg} cells reside, as not all tissues express high levels of IL-7 (REF. 31).

Defining memory T_{Reg} cells by epigenetics. Shifts in the epigenetic landscape and in transcriptional signatures may represent complementary approaches for identifying memory T_{Reg} cells. Fully activated and

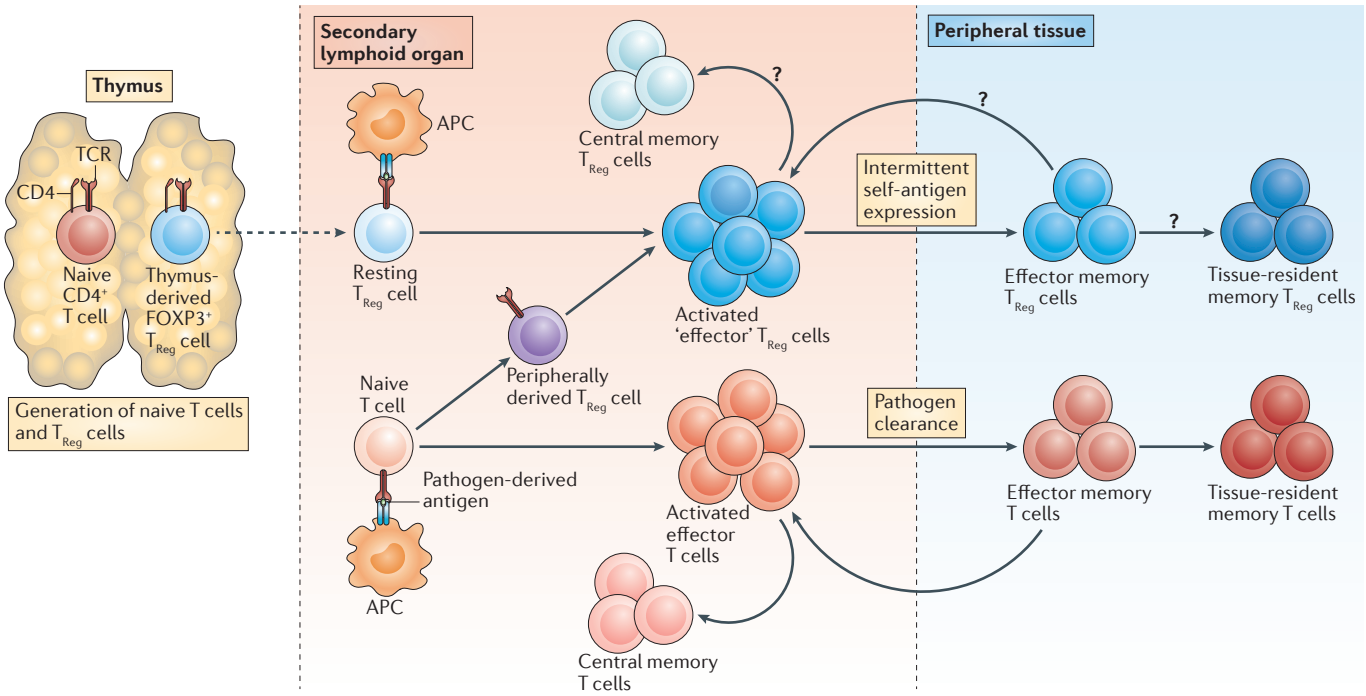

Figure 1 | **Life cycle of regulatory and conventional CD4⁺ T cells.** Naive conventional CD4⁺ T cells and regulatory T (T_{Reg}) cells are generated in the thymus. Upon antigen-specific activation in secondary lymphoid organs, these populations give rise to conventional effector T cells and 'effector' T_{Reg} cells. Effector T_{Reg} cells can arise from both thymus-derived and peripherally derived T_{Reg} cells. Central memory T cells are generated from a subset of activated conventional effector T cells and remain in secondary lymphoid organs. It is currently unknown whether central memory T_{Reg} cells are generated. Effector memory cells are generated from a subset of both conventional T cells and T_{Reg} cells. These cells migrate to antigen-expressing peripheral tissues where they stably reside (as tissue-resident memory T cells) or where they recirculate between blood and non-lymphoid tissues (as effector memory T cells). It is currently unknown whether the memory T_{Reg} cell populations that are found in peripheral tissues comprise tissue-resident memory T_{Reg} cells, effector memory T_{Reg} cells or both of these subsets. APC, antigen-presenting cell; FOXP3, forkhead box P3; TCR, T cell receptor.

lineage-committed T_{Reg} cells have defined epigenetic marks in *FOXP3* (REF. 32). For example, demethylation of a conserved intronic regulatory element in *FOXP3* (termed the conserved non-coding sequence 2 (CNS2) locus) is required for the maintenance of FOXP3 expression and for T_{Reg} cell stability upon exposure to inflammatory cytokines[33]. Furthermore, T_{Reg} cells activated in specific T_H-skewing environments express transcription factors that are also expressed by the effector CD4⁺ T cell lineage they most potently suppress[34–36]. For example, T_{Reg} cells that preferentially suppress T_H1 cell responses express T-bet, the canonical transcription factor that promotes T_H1 cell differentiation[35]. Thus, it is conceivable that prior activation or differentiation in memory T_{Reg} cells can be identified by epigenetic markers that are indicative of stable and open *FOXP3* expression in addition to transcriptional regulators that control effector CD4⁺ T cell differentiation.

Challenges in defining memory T_{Reg} cells. Perhaps the greatest challenge in defining memory T_{Reg} cells has been a lack of evidence that T_{Reg} cells can persist for prolonged periods of time in the absence of antigen. In the thymus, maturing thymocytes that express T cell receptors (TCRs) that have fairly high affinity for self peptide–MHC complexes differentiate into thymus-derived T_{Reg} cells[37] (BOX 1). Thus, it is inferred that most, if not all, T_{Reg} cells have specificity for self antigen. In turn, given that most self antigens are constitutively expressed, a challenge in defining memory T_{Reg} cells is identifying cells of defined specificity that persist in the absence of cognate antigen. One approach to address this issue has relied on mouse models in which expression of surrogate self antigens in tissues can be precisely turned on and off, allowing identification of antigen-specific T_{Reg} cells that persist after antigen expression is extinguished[11,13,29]. In addition, there are T_{Reg} cells that recognize foreign microbial antigens expressed by pathogens that cause acute transient infection, facilitating the identification of pathogen-specific T_{Reg} cells that persist after the infection resolves[14,15]. These models have been instrumental in defining memory T_{Reg} cells and are discussed in detail below.

Intricately associated with the difficulty in testing whether T_{Reg} cells can persist in the absence of antigen stimulation are challenges in showing that these cells respond more robustly upon repeated antigen exposure. For memory effector T cells, this is measured by the kinetics and the magnitude of proliferation and effector cytokine production, as well as by the kinetics of pathogen clearance. However, these criteria cannot be used for memory T_{Reg} cells. T_{Reg} cells secrete a limited repertoire of cytokines, most of which are difficult to quantify on

Box 1 | **Regulatory T cell subsets**

Forkhead box P3 (FOXP3)-expressing regulatory T (T_{Reg}) cells can be separated into several subsets on the basis of the sites in which they are generated, their relative differentiation state and the tissues in which they primarily reside. Cells derived in the thymus are often termed 'natural' T_{Reg} cells, and those derived outside of the thymus are often referred to as 'induced' or 'adaptive' T_{Reg} cells. There has been a consensus to rename these subsets 'thymus-derived' T_{Reg} cells (tT_{Reg}) and 'peripherally-derived' T_{Reg} cells (pT_{Reg}), respectively[102]. Naive or resting T_{Reg} cells are those that have yet to encounter their cognate antigen in the periphery or those that are constantly being exposed to antigen but the interactions are below the threshold for full activation. By contrast, effector T_{Reg} cells are cells that have received strong antigen stimulation outside of the thymus and have become fully activated, reflected by their proliferative index, changes in surface markers and enhanced suppressive function. Memory T_{Reg} cells have responded to antigen and are capable of surviving for fairly long periods of time even in the apparent absence of antigen (FIG. 1). T_{Reg} cells in tissues have different phenotypes and functional capacity compared with those found in secondary lymphoid organs and peripheral blood. Specialized populations of these cells have been identified in visceral adipose tissue, muscle, the gastrointestinal tract and skin (reviewed in REFS 38,40).

a per cell basis and may differ with respect to the tissues in which the cells reside[38]. The best criterion for functional changes associated with T_{Reg} cell memory is enhanced cell-intrinsic suppressive capacity and this is often difficult to measure with precision.

Early evidence for memory T_{Reg} cells. Despite inherent caveats in their phenotypic and functional characterization, there are several lines of evidence that support the existence of memory T_{Reg} cells. The first report of memory in a regulatory (also known as suppressor) T cell population was almost four decades ago[39]. Using an immunization approach with haptenated human IgG, Loblay and colleagues[39] showed that suppressor cells were generated in the T cell compartment upon primary exposure to antigen. Using adoptive transfer experiments, they went on to show that these cells were long-lived (at least 9 months) and suppressed immune responses with accelerated kinetics upon secondary challenge. In addition, during the secondary response, far fewer of these suppressor cells (5–10-fold fewer) were required to achieve a level of suppression equivalent to that observed in primary responses. These authors postulated that memory suppressor cells could have an important role in maintaining long-lived tolerance to self antigens. Although this work introduced the concept of regulatory memory, experiments during this time were considerably hampered by the lack of markers to isolate and functionally characterize suppressor cell populations. However, the discovery of FOXP3 as a lineage-defining marker for T_{Reg} cells has enabled more precise analyses of regulatory cell populations[9]. A wealth of phenotypic and functional characterization of T_{Reg} cells has emerged, and the potential for memory in this compartment has been recently revisited using several experimental models (FIG. 2).

Memory T_{Reg} cells with self antigen specificity

Many early studies of T_{Reg} cell biology focused on how these cells are generated in the thymus and the mechanisms by which they function in secondary lymphoid tissues. However, it has become increasingly appreciated that immune suppression by T_{Reg} cells is also required to regulate inflammation in non-lymphoid tissues. In turn, T_{Reg} cells recovered from different tissues seem to have distinct functional properties[38,40]. To investigate the nature of T_{Reg} cell responses in the skin, transgenic mice were generated in which a defined model antigen could be inducibly expressed in keratinocytes[11]. In this model, expression of the model antigen was constitutive in the thymus but tightly regulated in the skin, mimicking the expression pattern of tissue-restricted self antigens. Importantly, antigen expression in skin could also be silenced, allowing characterization of antigen-specific memory T cells that persist without ongoing exposure to cognate antigen. As expected, constitutive expression in the thymus resulted in the generation of a large population of antigen-specific T_{Reg} cells that seeded all secondary lymphoid organs. Upon antigen induction in the skin, these cells robustly proliferated, increased expression of T_{Reg} cell-intrinsic molecules that mediate immune suppression (such as CTLA4) and migrated to the skin to resolve the inflammatory response mediated by antigen-specific effector T cells[11]. Although few antigen-specific T_{Reg} cells were present before the induction of antigen expression, a distinct population that retained high levels of CTLA4 expression persisted in the skin long after antigen expression had been turned off. Upon re-expression of antigen (analogous to a secondary response), skin inflammation was attenuated and resolved with accelerated kinetics compared with the primary response. Depletion of T_{Reg} cells in the interval between initial and subsequent antigen expression ameliorated these beneficial effects against skin disease. This was the first evidence that antigen-specific $FOXP3^+$ T_{Reg} cells could fulfil immunological criteria for memory and persist as bona fide memory cells.

In most TCR-transgenic systems, the α-chain of the expressed TCR can pair with endogenous TCR α-chains[41]. This results in more than one TCR specificity being expressed on a single transgenic T cell, giving the cell the potential to recognize multiple different antigens. This caveat is circumvented by breeding TCR-transgenic mice onto a recombination-activating gene (RAG)-deficient background. In this setting, endogenous TCR chains are not expressed and thus cannot combine with transgenic TCR chains, which results in the production of T cells that bear only the transgenic TCR. As experiments in the inducible skin antigen system described above used TCR-transgenic T cells on a RAG-sufficient background, it is conceivable that T_{Reg} cells that persisted after cessation of antigen expression were maintained in the tissue through continued recognition of self antigen by alternative TCRs. To circumvent this caveat and to definitively test whether T_{Reg} cells could persist in tissues in the absence of antigen, an adoptive transfer approach was used, in which TCR-transgenic T cells on a RAG-deficient background were transferred into recipient mice capable of induced antigen expression in skin[13]. Antigen was induced for only 7 days and then extinguished. Consistent with previous results, a subset of T_{Reg} cells persisted in the skin for at least 60 days after cessation of antigen expression and had a low basal rate of proliferation, properties that are

Tissue-restricted self antigens
Self antigens that are expressed in specific tissues during defined periods of time. Hair follicle-associated antigens are an example of tissue-restricted self antigens in skin.

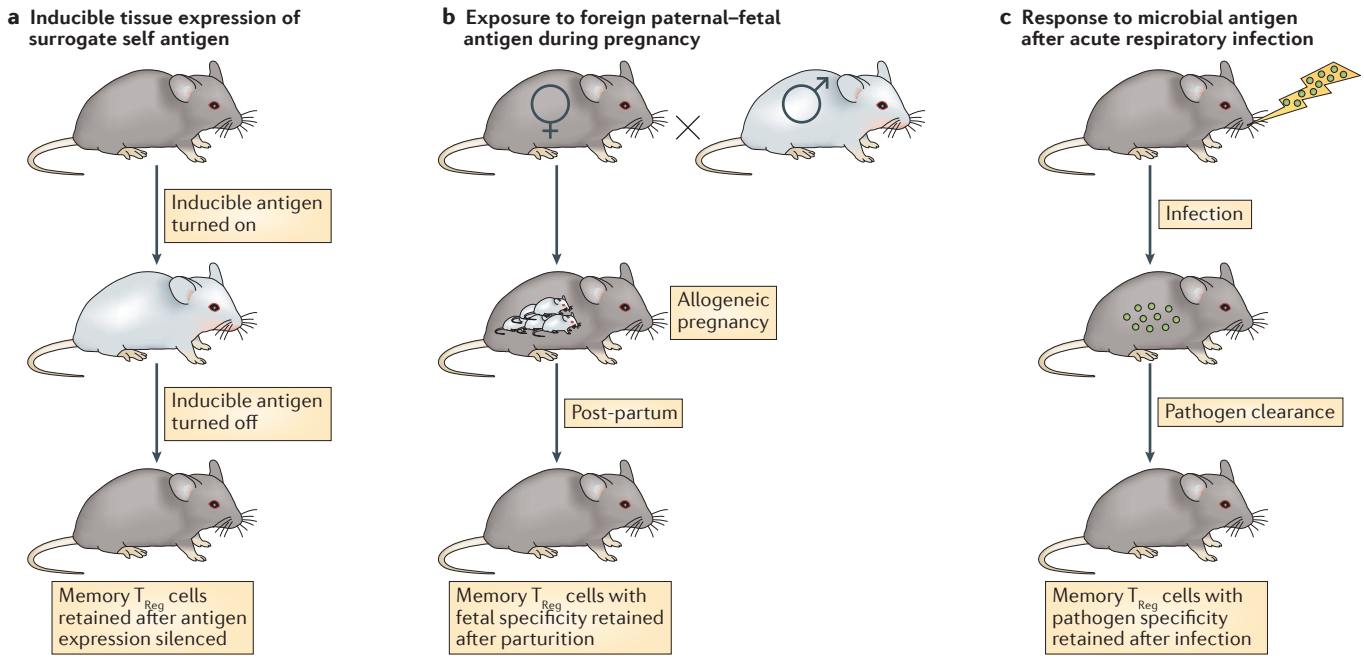

Figure 2 | **Mouse models for studying memory T_{Reg} cells.** Three primary mouse models have been used to identify and to characterize memory regulatory T (T_{Reg}) cells. **a** | In a tissue-specific inducible antigen model, expression of a pseudo-self antigen can be precisely turned on and off. This system facilitates the generation of memory T_{Reg} cells (by turning on the antigen) and their isolation and characterization in both secondary lymphoid organs and peripheral tissues after the antigen is turned off[11,13]. **b** | In an antigen-specific gestational model, maternal $CD4^+$ T cells with surrogate fetal specificity can be precisely identified during primary pregnancy, post-partum and with fetal antigen restimulation in subsequent pregnancies[12]. **c** | In an acute infection model with influenza virus, the initial infection is rapidly cleared but virus-specific memory T_{Reg} cells are generated and maintained long term. These memory T_{Reg} cells mitigate the tissue damage that occurs upon reinfection with the virus[14,15].

characteristic of tissue memory cells[42]. Moreover, in this model, all T_{Reg} cells are generated peripherally (that is, outside of the thymus) as there are no thymus-derived T_{Reg} cells present in the initial inoculum, which suggests that memory T_{Reg} cells can be generated *in vivo* from a peripherally generated T_{Reg} cell population.

Memory T_{Reg} cells in antimicrobial host defence

The finding that high affinity for self antigen has a major role in generating T_{Reg} cells in the thymus suggests that most, if not all, thymus-derived T_{Reg} cells are specific for self. However, several studies have identified T_{Reg} cells that recognize pathogen-derived peptides and some of these T_{Reg} cells seem to be generated in the thymus[43–45]. The ability to identify and to track T_{Reg} cells that are specific for foreign microorganisms offers a more conventional setting in which memory T_{Reg} cells can be identified. In a model of acute lung infection with influenza, virus-specific T_{Reg} cells increased 50-fold during the primary infection[15]. Virus-specific T_{Reg} cells expressed low levels of L-selectin but did not differ from resting T_{Reg} cells with respect to CD44 expression (which, as discussed above, is constitutively expressed at high levels even on resting T_{Reg} cells). Similar to effector T cell populations, the majority of virus-specific T_{Reg} cell populations also contracted after resolution of the primary infection. However, a small fraction of antigen-specific T_{Reg} cells persisted for more than 50 days after infection, representing a surviving population of memory cells. Upon reinfection, the virus-specific memory T_{Reg} cell pool underwent a 10-fold expansion that closely mirrored the expansion of the memory effector T cell population in kinetics and magnitude. Moreover, memory T_{Reg} cells significantly suppressed effector T cell population expansion and cytokine production in both systemic and tissue-specific models of reinfection[15]. In addition, they mitigated tissue damage without compromising viral clearance. These results were essentially repeated by a different group using a very similar model of infection[14]. Taken together, this work supports the hypothesis that memory T_{Reg} cells are generated to regulate potent memory effector responses and to thwart collateral damage to tissues that occurs with robust immune stimulation during infection[39]. Nonetheless, how memory T_{Reg} cells attenuate tissue inflammation without compromising pathogen clearance remains to be determined.

Memory T_{Reg} cells in maternal–fetal tolerance

Among genetically distinct individuals in naturally occurring outbred populations, pregnancy requires maternal tolerance to genetically foreign paternal antigens expressed by the developing fetus. Multiple mechanisms have evolved to establish and to reinforce fetal tolerance to protect immunologically foreign fetal tissue

from maternal rejection. One such mechanism is the systemic expansion of maternal T_{Reg} cell populations, which primarily comprise FOXP3$^+$ cells with defined fetal specificity. Reciprocally, reduced expansion of these maternal T_{Reg} cell populations has been widely associated with human pregnancy complications including pre-elampsia, premature birth and spontaneous abortion[46,47]. However, the fate of these T_{Reg} cells after parturition is not clear. Are they lost shortly after birth and generated *de novo* with each pregnancy or are they maintained for long periods of time in the non-pregnant state? If they persist, would they confer a reproductive advantage by reinforcing fetal tolerance in subsequent pregnancies?

To address these questions, the differentiation of maternal CD4$^+$ T cells with specificity for a defined surrogate fetal antigen was evaluated after primary pregnancy and in response to fetal antigen restimulation in subsequent pregnancies[12]. Fetus-specific maternal CD4$^+$ T cells accumulated throughout pregnancy and persisted at increased levels for the first 100 days after parturition. However, the level of maternal T_{Reg} cells with fetal specificity also progressively diminished within this post-partum time frame, similarly to the reduction in numbers of antigen-specific effector CD4$^+$ T cells with antigen elimination after acute infection. Re-exposure to the same fetal antigen in subsequent pregnancies primed previously generated memory T_{Reg} cell populations to re-expand with accelerated kinetics compared with the initial pregnancy. In turn, the highly enriched pool of fetus-specific T_{Reg} cells in secondary pregnancy compared with primary pregnancy conferred remarkable protective properties against disruptions in fetal tolerance[12]. These findings suggest that memory T_{Reg} cells in mothers reinforce fetal tolerance and establish an immunological basis for partner-specific protection against complications in secondary compared with primary human pregnancies[48,49].

The immunological parameters controlling post-partum retention of maternal T_{Reg} cells with pre-existing fetal specificity remains to be determined. However, the ubiquitous engraftment of genetically distinct fetal cells in mothers after pregnancy opens up the intriguing possibility that microchimeric fetal cells may provide a source of cognate antigen required for sustaining the accumulation of maternal memory T_{Reg} cells[50,51]. In other words, pregnancy-induced maternal T_{Reg} cells may not represent bona fide memory cell but may instead be maintained by antigenic stimulation from fetal cells that establish microchimerism in mothers after pregnancy[52]. This idea is consistent with recent findings that microchimeric maternal cells retained in offspring prime the sustained increase in T_{Reg} cells with non-inherited maternal antigen specificity[53]. Compulsory early developmental exposure to genetically foreign maternal antigens primes in offspring sustained tolerance to non-inherited maternal antigens and persistently increased T_{Reg} cells with specificity to these genetically foreign maternal antigens[54–56]. Conversely, targeted depletion of microchimeric maternal cells in offspring causes a rapid and precipitous decline in the increased accumulation of T_{Reg} cells with non-inherited maternal antigen specificity[53]. Whether these T_{Reg} cells are phenotypically more consistent with effector or memory T_{Reg} cells remains to be determined. Nevertheless, these results imply that some T_{Reg} cell subsets may require persistent cognate antigen stimulation for long-term numerical persistence, which is analogous to the necessity of antigen exposure reminders for numerical and functional maintenance of effector CD4$^+$ T cells with microbial specificity[57–59].

Evidence for memory T_{Reg} cells in humans

The mouse models described above define memory T_{Reg} cells phenotypically and functionally in various biological settings. In all cases, the approach used relied on inducing and analysing cells specific for defined antigens in a highly controlled *in vivo* environment. The inherent complexity of carrying out clinical experiments and the limited availability of tools for tracking T_{Reg} cells of defined antigen specificity precludes these types of studies in humans. Instead, studies of human memory T_{Reg} cells have mostly relied on phenotypic characterization and *in vitro* assays. Human T cells express the RO isoform of CD45 in the thymus and convert to CD45RA upon emigration to peripheral tissues[60,61]. Upon antigen recognition in the periphery, these cells switch back to CD45RO. Almost all CD45RA-expressing CD4$^+$ T cells *in vitro* lose CD45RA expression and transition to CD45RO$^+$ cells after 4 days of TCR stimulation[62]. Thus, circulating human T cells are often termed 'memory' cells if they express the CD45RO isoform. Although this marker is commonly used to distinguish naive T cells from memory T cells, it should be noted that expression of CD45RO alone does not define a T cell as being a bona fide memory cell. This marker does not distinguish between cells that persist in the absence of antigen and those that are continually being exposed to antigen. In addition, isoform switching from CD45RO back to CD45RA in the periphery has been reported[63]. Nevertheless, expression of CD45 isoforms together with chemokine receptors and selectins is now widely used to distinguish naive and memory T cells in humans[64].

Using a combination of CD25, CD45RA and FOXP3 expression, Miyara and colleagues[65] showed that peripheral blood of healthy humans contains two phenotypically and functionally distinct subsets of T_{Reg} cells: CD45RA$^+$FOXP3^{low} and CD45RA$^-$FOXP3^{hi} cells, termed 'resting' and 'activated' T_{Reg} cells, respectively[65]. Both populations were stable, highly suppressive T_{Reg} cell subsets that lacked effector cytokine production. However, CD45RA$^-$FOXP3^{hi} T_{Reg} cells expressed higher levels of cell-intrinsic activation markers such as CTLA4, ICOS and HLA-DR. Resting T_{Reg} cells were highly prevalent in cord blood and expressed higher levels of CD31, which suggests recent emigration from the thymus, whereas activated T_{Reg} cells were reduced in cord blood and increased with age. In addition, resting T_{Reg} cells readily proliferated and converted to activated T_{Reg} cells upon stimulation *in vitro* and *in vivo*. Taken together, these results suggest that, in humans, naive or resting T_{Reg} cells emigrate from the thymus in early life and, upon encounter with antigen in the periphery, these cells proliferate and differentiate into 'activated' effector T_{Reg} cells. As the antigen specificity of these subsets was not determined,

it is not known whether activated T_{Reg} cells were constantly being exposed to their cognate antigen or whether these cells persist at expanded levels in the absence of cognate antigen stimulation. However, given the fact that these results correlate well with the persistence of T_{Reg} cells with specificity for tissue-restricted self antigens that have been defined in mouse models, it is conceivable that a subset of activated T_{Reg} cells in human peripheral blood mononuclear cells are memory T_{Reg} cells that persist and remain activated in the absence of ongoing antigen stimulation.

Several reports show that CD45RA$^+$ T_{Reg} cells progressively decline in peripheral blood with age, accompanied by a reciprocal increase in CD45RO$^+$ T_{Reg} cells[62,66,67]. Human umbilical cord blood contains the highest percentage of CD45RA$^+$ T_{Reg} cells[66,68]. These results are consistent with the idea that CD45RA$^+$ T_{Reg} cells represent a resting population that convert to CD45RO$^+$ activated or memory T_{Reg} cells upon antigen exposure in peripheral tissues. Interestingly, antigen-experienced CD45RA$^-$ T_{Reg} cells can be further subdivided on the basis of HLA-DR expression[68]. Distinct populations of CD45RA$^-$HLA-DR$^-$ and CD45RA$^-$HLA-DR$^+$ T_{Reg} cells are present in human peripheral blood, thymus and umbilical cord blood. Phenotypic and functional analysis of these populations revealed that they express a common 'core' T_{Reg} cell gene signature and are both highly suppressive. However, they differ with respect to their activation state, suppressive capacity and cytokine secretion. Compared with HLA-DR$^-$ T_{Reg} cells, HLA-DR$^+$ T_{Reg} cells expressed higher levels of T_{Reg} cell-associated activation markers, were more suppressive *in vitro* and produced lower levels of effector cytokines. Given the more differentiated phenotype observed in the HLA-DR$^+$ fraction, it is interesting to speculate that these cells might represent bona fide memory T_{Reg} cells. By contrast, HLA-DR$^-$ cells may be recently activated but not fully differentiated T_{Reg} cells. However, because HLA-DR has been shown to be expressed on recently activated conventional T cells in humans[69], it is possible that CD45RA$^-$HLA-DR$^+$ T_{Reg} cells represent recently activated 'effector' T_{Reg} cells and not memory T_{Reg} cells. Important next steps will be to establish the antigen specificity of these phenotypically distinct human T_{Reg} cell populations.

As a result of the technical limitations of analysing and isolating cells from non-haematopoietic tissues, the study of memory T_{Reg} cells in humans has mainly focused on peripheral blood cells. However, it is well known that a large fraction of memory cells reside within peripheral tissues and that blood may simply be a conduit for memory cells that are actively trafficking between tissues or between tissues and secondary lymphoid organs[16]. Thus, it is important to study human memory T_{Reg} cells in tissues in addition to blood. To this end, T_{Reg} cells have recently been isolated from human skin and phenotypically and functionally characterized[30]. It was found that almost all T_{Reg} cells in adult skin express CD45RO, whereas a considerable fraction of T_{Reg} cells in fetal skin lacked CD45RO expression and instead were CD45RA$^+$. In addition, T_{Reg} cells in adult skin express high levels of other markers associated with T cell memory, including CD27 and B cell lymphoma 2 (BCL-2). Compared with cutaneous memory effector T cells, memory T_{Reg} cells expressed unique TCR sequences, did not express CC-chemokine receptor 7 (CCR7) and failed to migrate out of skin *in vivo*. These results suggest that human skin contains T_{Reg} cells with an 'effector memory' phenotype that recognize unique antigens and that stably reside in this tissue. This population may be most similar to tissue-resident memory cells[16]. The factors required for maintaining these activated memory T_{Reg} cells in skin and the specific antigens that they recognize remain to be elucidated.

Generation and maintenance of memory T_{Reg} cells

How memory T_{Reg} cells are generated and maintained is a fundamentally important question and an area of active investigation. Several factors are likely to be involved — we discuss these below, and a summary of the ontogeny of memory T_{Reg} cells as well as salient features of resting, effector and memory T_{Reg} cell subsets is suggested in FIG. 3.

Cytokines. Specific cytokine growth factors probably have a role in the generation and the maintenance of memory T_{Reg} cells. Both IL-2 and IL-7 have been implicated in the generation and the maintenance of memory CD4$^+$ T cell subsets[70]. After acute infection with the intracellular bacterial pathogen *Listeria monocytogenes*, increased expression of CD25 (the high-affinity IL-2 receptor α-chain) was preferentially seen on bacteria-specific effector memory T cells, and cell-intrinsic genetic deficiency of CD25 significantly compromised the ability to generate this population[71]. In a separate model using TCR-transgenic cells, IL-2 signalling early after vaccination was required for IL-7 receptor (IL-7R) expression and for the generation of long-lived memory cells[72]. Given that IL-2-mediated signalling is important for the generation of effector memory cells and because IL-2 has a major role in T_{Reg} cell generation and maintenance[73], it follows that this pathway might also be important in the generation of memory T_{Reg} cells.

The α-chain of the receptor for IL-7, CD127, is expressed at high levels on CD4$^+$ effector memory T cells and has a major role in their maintenance in peripheral tissues[19]. The majority of T_{Reg} cells found in secondary lymphoid organs express low levels of IL-7R; however, memory T_{Reg} cells in skin have increased expression of IL-7R, which suggests that this pathway may be involved in maintaining these cells in tissues[29]. To dissect the relative contribution of the IL-2 and the IL-7 pathways in the generation and the maintenance of memory T_{Reg} cells, both genetic deletion and antibody-mediated neutralization approaches have been used in the aforementioned model of inducible antigen expression in skin[29]. Results from these studies showed that IL-2 was necessary for the generation of memory T_{Reg} cells, whereas IL-7 but not IL-2 was required for their maintenance in skin. Consistent with these results, Smigiel and colleagues[74] found that a subset of CD44^{hi}CD62L^{low}CCR7^{low} T_{Reg} cells have reduced levels of IL-2 receptor signalling and that IL-2 was not required to maintain these cells *in vivo*[74].

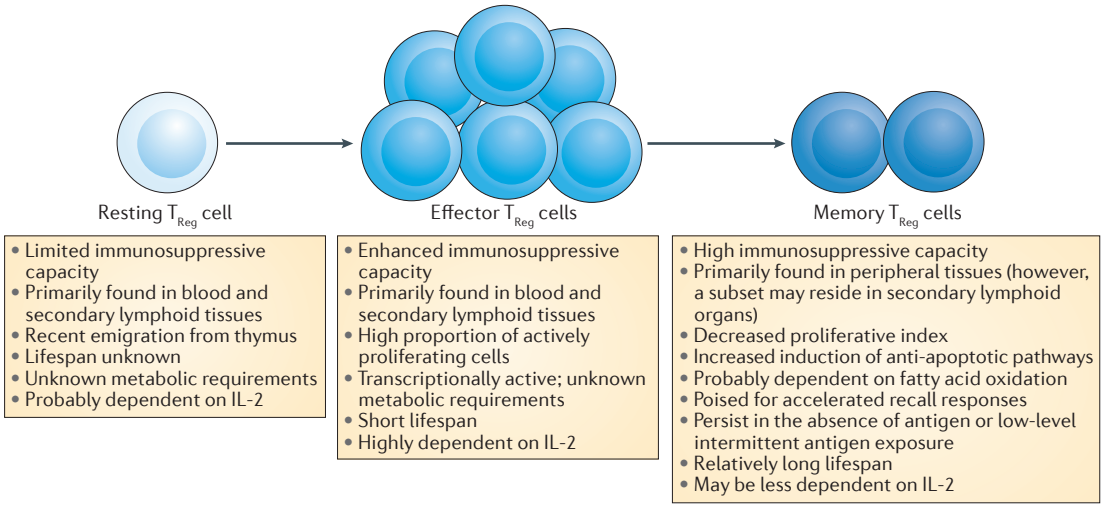

Figure 3 | **Predicted model for the relationship between resting, effector and memory T_{Reg} cells.** Regulatory T (T_{Reg}) cells that are derived in the thymus are maintained in the periphery in a resting or naive state. They are intermittently exposed to their cognate antigens but these T cell receptor–MHC interactions are below the threshold for full activation. Upon strong stimulation with antigen in secondary lymphoid organs, resting T_{Reg} cells are activated, proliferate and differentiate into effector T_{Reg} cells. A subset of effector T_{Reg} cells is capable of differentiating into long-lived memory T_{Reg} cells. Memory T_{Reg} cells can reside in non-lymphoid tissues and are capable of surviving in the absence of antigen or low levels of intermittent antigen exposure. The figure highlights key features of each population[11–15,29,30,53,65,74,84]. IL-2, interleukin-2.

Transcription factors. For $CD8^+$ T cells, several studies have identified transcripts that are uniquely expressed in memory cells[75–78]; however, lineage-defining genes that drive differentiation of long-lived memory subsets have not been defined. Thus, the prevailing view is that complex networks coordinately drive memory cell generation and stability. Relative levels of specific transcription factors and specific epigenetic landscapes seem to be major determinants. For example, high levels of the transcription factor T-bet with concomitant low levels of the transcription factor eomesodermin (EOMES) promote the differentiation of naive $CD8^+$ cells into short-lived effector cells, whereas low levels of T-bet and high levels of EOMES drive their development into memory cells[79,80]. The same paradigm seems to hold true for $CD4^+$ T cells. The transcription factors B lymphocyte-induced maturation protein 1 (BLIMP1; also known as PRDM1) and BCL-6 have been shown to coordinately influence the development of effector and memory $CD4^+$ T cell fates[81,82]. Interestingly, high levels of BLIMP1 are expressed in a subset of T_{Reg} cells with an 'effector' phenotype and in follicular T_{Reg} cells, which also express BCL-6 (REFS 83,84). Thus, it is interesting to speculate that gradients of specific transcription factors drive effector and memory T_{Reg} cell fates, similarly to what is seen in other $CD4^+$ T cell subsets.

Epigenetic modifications. Although differential transcription factor expression will probably have a major role in promoting memory T_{Reg} cell development, binding of these factors to the appropriate DNA elements will be crucial. Chromatin accessibility and specific epigenetic modifications are proving to be major determinants of T cell memory fate[24,85,86]. How chromatin is assembled and modified early after T_{Reg} cell activation will probably differ between cells that are destined to be short-lived effectors and long-lived memory T_{Reg} cells. Elucidating these differences and the mechanisms by which they are established is central to our understanding of how memory T_{Reg} cells are generated and maintained. An assay for transposase-accessible chromatin using sequencing (ATAC-seq) is currently being used to map chromatin accessibility across the entire genome with great resolution using very small numbers of cells[87]. This technology may be well suited to discover how epigenetic landscapes differ between naive and memory T_{Reg} cell subsets and has the potential to reveal which transcription factors bind at specific enhancer regions in these cells.

Metabolic pathways. Different metabolic pathways are used during different stages of T cell activation and differentiation. It is now well understood that the metabolic demands of resting T cells are quite different from cells that are actively proliferating and mediating effector functions in the face of an ongoing immune response. Memory $CD8^+$ T cells use unique metabolic pathways compared with both naive and short-lived effector cells. Whereas proliferating effector cells rely more on aerobic glycolysis, memory cells are dependent on fatty acid oxidation[88,89]. Sustained glycolytic activity inhibits the formation of memory, whereas inhibiting glycolysis promotes the development of memory cells[90,91]. Consistent with this, inhibition of mammalian target of rapamycin (mTOR) promotes fatty acid oxidation and increases the formation of memory cells[92]. It is hypothesized that memory T cells rely on fatty acid oxidation because it affords a greater capacity to generate energy under

stress and enables a more rapid response upon reinfection[93]. Interestingly, the same metabolic pathways that promote T cell memory also promote the development of T_{Reg} cells. Compared with effector T cells, T_{Reg} cells express lower levels of glucose transporter 1 (GLUT1; also known as SLC2A1) and have higher basal lipid oxidation rates, which suggests that they primarily rely on fatty acid oxidation for their energy requirements[94]. Consistent with this, blocking either glycolysis or mTOR signalling promotes T_{Reg} cell development[95–97]. Given the metabolic similarities between memory effector cells and T_{Reg} cells, it has been suggested that activated naive cells differentiate into effector T_{Reg} cells if mTOR signalling is high and into long-lived memory T_{Reg} cells in the presence of low levels of mTOR activating signals[98,99]. Local levels of transforming growth factor-β (TGFβ) are thought to have a role in this process[98]. Taken together, these studies support the idea that the metabolic requirements for T_{Reg} cells differ from those of other $CD4^+$ T cell populations. However, it is currently unknown how metabolism differs between specific T_{Reg} cell subsets. Whether memory T_{Reg} cells can be distinguished from naive and effector T_{Reg} cells on the basis of different metabolic requirements (that is, glycolysis versus fatty acid oxidation) remains to be determined.

Unanswered questions and future directions

Functional studies in mice and complementary studies with human cells and tissues have identified the existence of FOXP3-expressing memory T_{Reg} cells. However, our current understanding of memory T_{Reg} cell biology is rudimentary compared with that of memory effector T cells. This primarily stems from the very recent identification of memory T_{Reg} cells and the lack of memory-specific phenotypic markers for identifying these cells. Fundamental questions remain to be answered, with perhaps the first being, are there specific markers that can reliably separate memory T_{Reg} cells from short-lived 'effector' T_{Reg} cells and resting T_{Reg} cells? Comprehensive gene-expression profiling combined with flow cytometric characterization of memory T_{Reg} cells in mouse models in which these cells can be reliably generated and purified will be required to elucidate a 'core' memory T_{Reg} cell signature. Contrasting this profile with that of purified resting and recently activated effector T_{Reg} cells will probably establish a set of parameters that can more reliably identify memory T_{Reg} cells in both mice and humans. As heterogeneity will probably exist in seemingly pure T_{Reg} cell populations, single cell expression analysis may be required to more precisely define these subsets. To determine whether memory T_{Reg} cells consist of central, effector and tissue-resident subsets, this analysis will need to be carried out on cells isolated from both secondary lymphoid organs and peripheral tissues. Once candidate markers are identified, crucial next steps include investigating which represent true lineage-defining indicators of cell 'fate' versus those that reflect a transient cell 'state' influenced by local inflammatory signals — a task currently plaguing the much more mature memory $CD8^+$ T cell field[1]. In addition, it will be important to discern whether memory exists in other regulatory immune populations, such as type 1 regulatory T (T_R1) cells[100] and regulatory B cells[101]. It is conceivable that these cells work together with FOXP3-expressing memory T_{Reg} cells to promote self tolerance and to maintain immune homeostasis.

The identification of T_{Reg} cells as a dedicated immune suppressive $CD4^+$ T cell lineage and the essential role that these cells have in maintaining immune homeostasis has raised many exciting new questions regarding the fundamental biology of these cells. These efforts are beginning to bear fruit, as novel treatment strategies aiming to either augment or to inhibit T_{Reg} cells are beginning to enter the clinic to treat human disease. We now appreciate that T_{Reg} cells are heterogeneous, comprised of multiple subsets that differ depending on the tissues in which they reside and on their differentiation state. Comprehensively defining these subsets, both phenotypically and functionally, may result in new and more targeted therapeutic strategies. We are in the early stages of dissecting the biology of memory T_{Reg} cells and the potential role that they have in health and disease. However, the highly suppressive nature of these cells and the exciting potential for their persistence as memory cells make them promising candidates for therapeutic manipulation in a range of clinical settings (for example, transplantation, autoimmunity, microbial immunity and maternal–fetal medicine) in which the balance between immune stimulation and suppression requires more stringent regulation.

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Competing interests statement

The authors declare no competing interests.