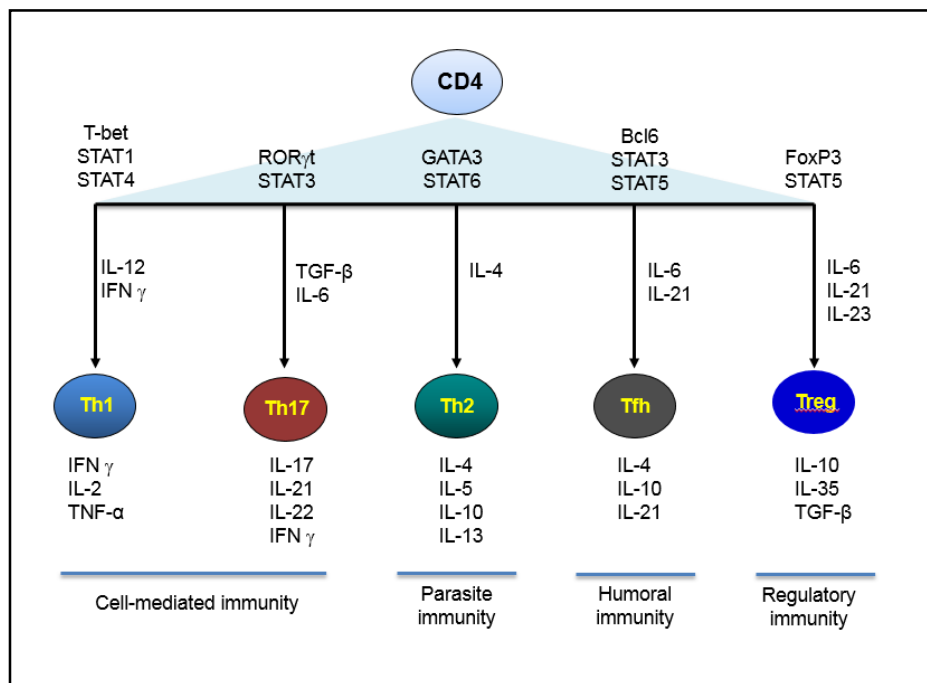




### T Cell Heterogeneity and Nomenclatures

The discovery and dissection of the functional differences between effector and memory T cell subsets have significantly advanced our understanding of the mechanisms controlling the development of T cell heterogeneity. Prior to activation by APCs, both CD4 T cells and CD8 T cells are designated as naïve and are maintained in a quiescent state. Following activation, T cells undergo programmed proliferation and differentiation, producing multiple lineages of effector T cells based on the production of distinct effector molecules [18,28]. Activated CD4 T cells can differentiate into distinct lineages of effector cells (**Figure 1**), such as T helper-1 (Th1), Th2 and Th17 and regulatory T cells (Tregs). Th1 CD4T cells are characterized by production of interferon- $\gamma$  (IFN- $\gamma$ ), whereas Th2 CD4 T cells secrete interleukin-4 (IL-4), IL-5 and IL-13 [18,20,29]. Th17

CD4 T cells are characterized by their capacity to produce high amounts of IL-17 and IL-21 [18,28]. CD4 T cells can also differentiate into Tregs, which can repress inflammatory T cells through the production of IL-10 and TGF- $\beta$  [30]. CD4 T cells may also differentiate into other subsets such as T follicular helper cells (Tfh) [31] and Th9 cells [32,33]. Tfh primarily reside in B-cell follicles and contribute to humoral immunity [31]. Th9 cells, which display an interesting plasticity, may act with Th2 in inflammatory responses or display immunosuppressive function through production of IL-10 [32,34,35]. Activation of naïve CD8 T cells mainly induces the generation of cytotoxic lymphocytes (CTLs) that produce IFN- $\gamma$  and cytotoxic molecules such as granzyme B (GZMB), perforin (PRF1), and Fas ligand (FASL). CD8 CTLs are capable of direct cell-mediated killing of target cells [5,6].



**Figure 1. Naïve CD4 T Cells Differentiate into Distinct Effector Subsets.** Following recognition of a specific antigen presented on an APC, naïve CD4 T cells become activated to differentiate and rapidly expand into distinct T effector subsets. Transcription factors, along with soluble factors present in the extracellular environment are key mediators of changes in T cell transcriptional programs that trigger T effector polarization. T effector subsets are classified by transcription factors that dominantly drive their phenotype as well as the cytokines they express.

CD4 and CD8 T cells both possess the ability to form immunological memory through differentiation into a population of antigen-specific memory T cells that persist throughout the lifetime of an individual after resolution of inflammation [17, 36, 37]. Following re-encounter with a specific antigen, memory T cells can quickly expand and elaborate effector function, thus providing the immune system with long-term protection against secondary antigen

encounters. Memory CD8 T cells are heterogeneous populations and have distinct capabilities in the context of providing long-term protection against tumor formation. They can be broadly classified into four subsets based on their tissue homing capacity, self-renewal capability, effector recall responsiveness and surface phenotype (**Figure 2**): effector memory T cells ( $T_{EM}$ ), central memory T cells ( $T_{CM}$ ), resident memory T cells ( $T_{RM}$ ), and stem cell-like memory T cells ( $T_{SCM}$ ) [3,8,38-44].  $T_{EM}$  express low

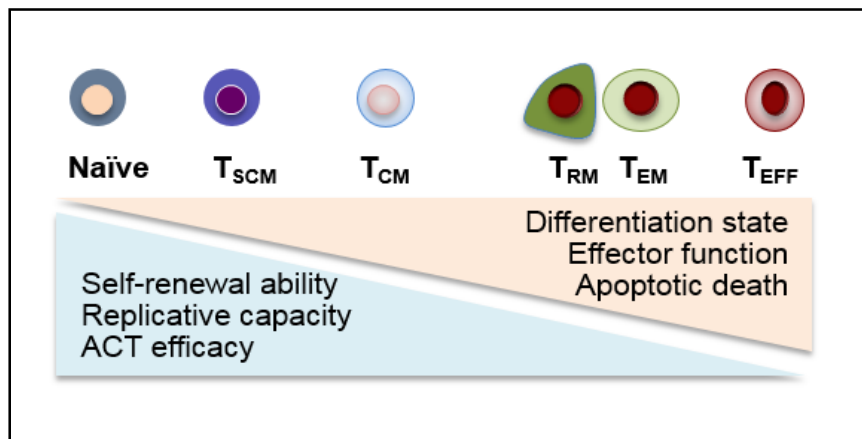
levels of CD62L and CCR7, allowing them to circulate and preferentially home to non-lymphoid tissues. T<sub>CM</sub> express CD62L and CCR7, restraining their homing to lymphoid tissues. T<sub>RM</sub> predominantly reside in the local non-lymphoid tissues, such as the brain, mucosa, lung and skin [7,39]. T<sub>RM</sub> express CD69 and CD10, surface markers, which distinguish them from T<sub>EM</sub> [7,45-48]. Finally, T<sub>SCM</sub> are a memory cell subset expressing a naïve cell-like phenotype of CD44<sup>low</sup>CD62L<sup>high</sup>Sca-1<sup>high</sup>CD122<sup>high</sup>Bcl2<sup>high</sup>. They possess the ability to differentiate into all subsets of memory CD8 T

cells and effector cells, while maintaining self-renewal capabilities [41,42]. Immunological memory mediated by CD8 and CD4 T cells is critical for prolonged protection against antigen reencounter and tumor formation (Figure 3). The functional complexity of effector and memory subsets characterize T cell heterogeneity. Transcription factors, which critically regulate differentiation into these subsets, play a fundamental role in programming the diverse functions of T cells, which collectively contribute to a comprehensive immune response.

	Naïve	T <sub>SCM</sub>	T <sub>CM</sub>	T <sub>EM</sub>	T <sub>RM</sub>	T <sub>EFF</sub>
<b>Human</b>	CD45RA <sup>+</sup> CCR7 <sup>+</sup> CD95 <sup>-</sup> CD122 <sup>-</sup> CD62L <sup>+</sup> CD69 <sup>-</sup> CD103 <sup>-</sup>	CD45RA <sup>+</sup> CCR7 <sup>+</sup> CD95 <sup>+</sup> CD122 <sup>+</sup> CD62L <sup>+</sup> CD69 <sup>-</sup> CD103 <sup>-</sup>	CD45RO <sup>+</sup> CCR7 <sup>+</sup> CD95 <sup>+</sup> CD122 <sup>+</sup> CD62L <sup>+</sup> CD69 <sup>-</sup> CD103 <sup>-</sup>	CD45RO <sup>+</sup> CCR7 <sup>-</sup> CD95 <sup>+</sup> CD122 <sup>+</sup> CD62L <sup>-</sup> CD69 <sup>-</sup> CD103 <sup>-</sup>	CD45RO <sup>-</sup> CCR7 <sup>-</sup> CD95 <sup>+</sup> CD122 <sup>+</sup> CD62L <sup>-</sup> CD69 <sup>+</sup> CD103 <sup>+/-</sup>	CD45RO <sup>+</sup> CCR7 <sup>-</sup> CD95 <sup>+</sup> CD122 <sup>+</sup> CD62L <sup>-</sup> CD69 <sup>-</sup> CD103 <sup>-</sup>
<b>Mouse</b>	CD62L <sup>+</sup> CD44 <sup>-</sup> Sca-1 <sup>-</sup> KLRG <sup>-</sup> CD122 <sup>-</sup> Bcl-2 <sup>+/-</sup> CD69 <sup>-</sup> CD103 <sup>-</sup>	CD62L <sup>+</sup> CD44 <sup>-</sup> Sca-1 <sup>+</sup> KLRG <sup>-</sup> CD122 <sup>+</sup> Bcl-2 <sup>+</sup> CD69 <sup>-</sup> CD103 <sup>-</sup>	CD62L <sup>+</sup> CD44 <sup>-</sup> Sca-1 <sup>+/-</sup> KLRG <sup>+/-</sup> CD122 <sup>+</sup> Bcl-2 <sup>+</sup> CD69 <sup>-</sup> CD103 <sup>-</sup>	CD62L <sup>-</sup> CD44 <sup>+</sup> Sca-1 <sup>+</sup> KLRG <sup>+</sup> CD122 <sup>+</sup> Bcl-2 <sup>+/-</sup> CD69 <sup>-</sup> CD103 <sup>-</sup>	CD62L <sup>-</sup> CD44 <sup>+</sup> Sca-1 <sup>+</sup> KLRG <sup>+</sup> CD122 <sup>+</sup> Bcl-2 <sup>+/-</sup> CD69 <sup>+</sup> CD103 <sup>+/-</sup>	CD62L <sup>-</sup> CD44 <sup>+</sup> Sca-1 <sup>+</sup> KLRG <sup>+</sup> CD122 <sup>+</sup> Bcl-2 <sup>+/-</sup> CD69 <sup>-</sup> CD103 <sup>-</sup>

**Figure 2. Surface markers of Distinct Effector and Memory Subsets.**

Upon APC activation, naïve CD8 T cells become activated and differentiate into effector cells. During clonal expansion, effector cells polarize toward various memory subsets that are classified based on their tissue homing capacity, self-renewal capability and effector recall responsiveness. Each subset expresses distinct surface phenotype that facilitates their separation and characterization of biological properties.



**Figure 3. Heterogeneity of Memory CD8 T Cell Subsets Frames a Comprehensive Immune Response.** Memory CD8 T cell subsets possess varying degrees of effector function and stemness as a consequence of their differentiation state. Stem-cell like memory CD8 T cells retain properties of naïve T cells, allowing them to differentiate into all other CD8 T effector and memory subsets. Life-long homeostatic proliferation of these memory CD8 T cells confers long-lasting protection against secondary antigen encounters.

### Transcription Factors and Distinct Lineages of CD4 T Cells

Dozens of transcription factors critical for the generation of distinct lineages of effector and memory T cells have now been identified [18,49]. Seminal studies have demonstrated that these transcription factors are important for maintaining the plasticity and stability of effector CD4 T cells [18,28,29,50,51].

**Th1 cells.** Th1 CD4 T cells are important in mediating protection against pathogens and tumor cells. Importantly, Th1 cells also play a critical role in mediating various types of inflammation, such as type I diabetes, graft-rejection of transplanted organs, and graft-versus-host disease (GVHD), a complication of allogeneic hematopoietic stem cell transplantation [18,52-54]. Several transcription factors have been found to regulate CD4 Th1 cell differentiation, including T-bet, Eomes, Runx3, activator of transcription (Stat) 1 and Stat4 [18,28]. These factors cooperate to direct Th1 differentiation and to maintain the stability of differentiated Th1 cells.

T-bet is a master regulator of Th1 differentiation, with loss of T-bet leading to dramatically impaired production of Th1 cells during immune response. T-bet expression was found to be strongly dependent on signal transducer and Stat1, rather than on IL-12-dependent Stat4. Stat1 is activated by IFN- $\gamma$ , and T-bet expression further induces IFN- $\gamma$  production by differentiating cells, thereby amplifying T-bet expression and upregulating the expression of IL12R $\beta$ 2 [17,18]. CD4 T cells expressing high levels of IL12R $\beta$ 2 respond to IL12 produced by APCs, thus ensuring selective expansion of T cells differentiating towards Th1 effector function [17,18]. Stat4, which is induced by IL-12, is also positively regulated by IFN- $\gamma$  [55]. Activated Stat4 supports Th1 differentiation by further inducing the expression of IFN- $\gamma$ , IL12R $\beta$ , and T-bet [56,57]. The transcription factor Runx3 is upregulated upon CD4 T cell stimulation and also functions to amplify T-bet and IFN- $\gamma$  expression [58]. Furthermore, overexpression of Runx3 *in vitro* has been shown to promote and accelerate Th1 differentiation [59].

Recent studies have demonstrated that several other transcription factors, such as Zbtb7b (also called Th-POK) and the Notch effector RBP-j/CSL, may also contribute to the development of distinct lineages of effector CD4<sup>+</sup> T cells [20,21,28,60,61]. Eomesodermin (Eomes), another member of the T-box protein family, is dispensable for antigen-induced Th1 cell development and function, but may induce IFN- $\gamma$  production in CD4 T cells under non-polarizing conditions when T-bet is not upregulated [62]. Thus, T-bet and Eomes cooperate with each other to promote IFN- $\gamma$  production under different conditions.

Th1 cell differentiation occurs in parallel with the repressed production of inappropriate cytokines such as IL-4 and IL-17 [18]. It is through this mechanism that T-bet suppresses the development of both Th2 and Th17 cells. T-bet prevents Th2 cell differentiation by inhibiting transcription of IL-4, a signature Th2 cytokine, and by inhibiting the function of Gata3, a master regulator for Th2 cell differentiation [63]. T-bet can also interact with the promoter of ROR $\gamma$ t (which encodes ROR $\gamma$ t, a master regulator of Th17) to inhibit Th17 cell differentiation [64,65].

**Th2 cells.** Th2 cells primarily mediate the adaptive immune response to parasitic protozoa and helminths [18,66,67]. Th2 cells are also able to drive B cells to produce several subclasses of IgG and IgE antibodies. Furthermore, cytokines produced by Th2 cells activate eosinophils and mast cells, causing inflammatory damage to tissues including the lung and airway [68-70]. Gata3 and Stat6 are transcription factors critical for the induction of Th2-associated cytokines (i.e., IL-4, IL-5 and IL-13) [63]. GATA3 conditional knockout studies showed that GATA3 expression is required for Th2 differentiation [71]. In differentiated Th2 cells, continuous GATA3 expression is essential for maintaining production of IL-5 and IL-13, but not IL-4. Furthermore, Gata3 has a dual function in the repression of Th1 differentiation by antagonizing T-bet expression in proliferating CD4 T cells [63,71]. Stat6 is the major signal transducer in IL-4-mediated Th2 cell differentiation and is critical for the production of IL-4 in CD4 T cells, as demonstrated by the failure of STAT6-deficient CD4 T cells to develop into IL-4-producing cells *in vitro*. Stat6 activation is also necessary and sufficient for inducing high expression levels of GATA3 [18,28,72-74].

**Th17 cells.** The Th17 subset is characterized by production of IL-17 and is important in mediating responses to pathogens. Th17 cells have also been implicated as potent effectors of autoimmune diseases such as Crohn's disease, ulcerative colitis, rheumatoid arthritis and psoriasis [18,50,65,75]. Th17 cell differentiation requires two key transcriptional regulators: ROR $\gamma$ t and Stat3. Deficiency of ROR $\gamma$ t leads to profound interruption of Th17 cytokine expression, whereas forced expression of ROR $\gamma$ t induces the production of IL-17A and IL-17F, both of which mediate pro-inflammatory responses, but differ in the type and site of inflammation [76,77]. Stat3 plays an important role in Th17 cell differentiation by inducing ROR $\gamma$ t and by directly binding to IL-17A and IL-17F promoters [50,65,75]. In addition to positive regulation of Th17 differentiation by ROR $\gamma$ t and Stat, transactivation of ROR $\gamma$ t by Runx1 is also critical for induction of the Th17 subset [63,78,79]. In contrast, the Runx1/FOXP3 interaction or Runx1/T-bet collaboration leads to the interruption of Runx1-mediated transactivation of RORC, thereby repressing Th17 differentiation [63,78,79].

**Treg.** There are two major classes of CD4 Treg cells, including natural Treg (nTreg) and inducible Treg (iTreg), both of which sustain immune system homeostasis by mediating self-tolerance and modulating inflammation. nTregs develop in the thymus during thymopoiesis, and are therefore termed thymic Tregs, whereas iTregs can be induced in peripheral tissues during immune responses [80,81]. Both subsets require the expression of the transcription factor Foxp3, which may be used to characterize these subsets [80]. Mutations of the FOXP3 gene can prevent Treg development, causing the fatal autoimmune disease IPEX [82]. iTreg3, a novel subset recently identified in mice and humans, is noteworthy because unlike previously identified subsets, it does not express Foxp3. Furthermore, this subset mediates immunosuppressive effects via IL-35 rather than the canonical cytokines IL-10 and TGF- $\beta$  [83,84]. Several elegant papers have recently reviewed Treg biology and it will therefore not be discussed here [85,86].

### Transcriptional Regulation of Effector and Memory CD8 T Cells

**Effector differentiation and expansion.** Upon APC activation, antigen-specific CD8 T cells undergo a highly reproducible pattern of clonal expansion and differentiation. TCR and costimulatory signaling together with cytokines activate transcription programs important for regulating effector differentiation and expansion. T-bet and Eomes have been shown to function as master regulators for promoting CD8 effector T cell differentiation and function [26,87,88]. CD8 T cells lacking both T-bet and Eomes lose CTL identity and abnormally differentiate into IL-17-producing CD8 T cells that cause excessive neutrophil infiltration and a lethal inflammatory syndrome during LCMV infection. During acute response, T-bet and Eomes have cooperative and partially redundant effects on promoting CTL formation by inducing the expression of the cytotoxic molecules perforin and GZMB in activated CD8 T cells [87,88]. Importantly, effector CD8 T cells expressing high levels of T-bet are prone to terminal differentiation and become KLRG1<sup>hi</sup> short-lived effector cells (SLECs) [9]. During chronic infections, effector CD8 T cells expressing high levels of Eomes are susceptible to exhaustion and ultimately lose their ability to control chronic infection [89]. Interestingly, this demonstrates that the phenotype, function, and long-term fate of effector CD8 T cells are acutely sensitive to the relative ratio of T-bet and Eomes [89], yet the regulation of this ratio in activated T cells remains largely unknown.

Blimp-1 contributes to a transcriptional program that enhances CTL functions, such as migration to sites of inflammation and production of IFN- $\gamma$  and GZMB [90-93]. Animals with a CD8 T cell-specific deficiency in Blimp-1 have an impaired ability to clear influenza virus due to poor recruitment of virus-specific CD8 T cells to the lungs [93-

95]. However, high expression of Blimp-1 promotes terminal differentiation of CD8 SLECs and induces exhaustion of chronically activated CD8 T cells [91-94]. Thus, Blimp-1 has multiple roles in regulating effector T cell responses.

IFN regulatory factor 4 (Irf4) regulates CD8 T cell differentiation and expansion during acute infection [96,97]. While Irf4 is dispensable for early activation of CD8 T cells, it is important for effector differentiation and expansion [96,97]. Irf4 simultaneously promotes the expression and function of Blimp-1 and T-bet along with repressed genes that mediate cell cycle arrest and apoptosis. Selective deletion of IRF4 in peripheral CD8 T cells impairs antiviral CD8 T cell responses [96]. Irf4 also influences the expansion of SLECs at the peak time of infection, but has no effect on the rate of T cell contraction. This effect of Irf4 is associated with increased expression of Eomes and Tcf1 in CD8 T cells [96].

Several other transcription factors regulate the expansion of effector CD8 T cells. Inhibitor of DNA binding 2 (Id2), which is a member of the inhibitor of DNA-binding family, is required for the survival of effector CD8<sup>+</sup> T cells during early expansion phase [27,98]. More recent studies suggest that Id2 is especially important for the formation of terminal KLRG-1<sup>hi</sup>T<sub>EFF</sub> [99]. As compared to Id2, Id3 promotes the survival of T<sub>EFF</sub> later during effector expansion, in particular when effector cells develop into memory cells [27]. Enforced expression of Id3 has been shown to be sufficient to restore SLEC survival and enhanced recall responses [100]. These data suggest that while both Id2 and Id3 are critical to the survival KLRG-1<sup>hi</sup>SLECs, their effects occur at different stages of effector expansion. Although the precise mechanisms by which Id2 and Id3 regulate the survival and expansion of effector cells remain largely unknown, available data show that their pro-survival effects are likely associated with their regulation of anti-apoptotic genes (e.g., Bcl, Serpinb9 and Bcl2l11) and genomic stability, respectively [27,98-100].

Recent studies have demonstrated the importance of the transcription factor Bcl11b in antigen-dependent clonal expansion and cytolytic activity of CD8 T cells [101]. BCL11b deficiency was shown to have no impact on effector differentiation, but caused significantly decreased proliferation of antigen-activated T cells later during clonal expansion phase. BCL11b deficiency in CD8 T cells also leads to deregulation of CD8 co-receptor and Plcy, both of which contribute to the impaired responsiveness of activated T cells [101]. It will be interesting to investigate how these transcription factors are coordinated to regulate the survival and expansion of effector CD8 T cells in the environment where effector cells reside and execute function.

**Memory formation and maintenance.** Memory CD8 T cells are derived from proliferating T cells during the clonal expansion phase and may be classified into four different

subsets (**Figure 2**):  $T_{CM}$ ,  $T_{EM}$ ,  $T_{RM}$ , and  $T_{SCM}$  [3,8,38-44]. Identifying the differentiation pathways for heterogeneous memory T cell subset development following naïve T cell activation has been an area of active investigation [7]. In mice, these cells can be classified based on surface phenotype (e.g., CD62L, CD4, CD127 and KLGR-1) [3,5,7]. Genome-wide studies reveal that  $T_{SCM}$  express gene programs that resemble, but are distinguishable from naïve T cells, thus being considered less differentiated than other subsets of memory cells [102]. As compared to  $T_{CM}$ ,  $T_{EM}$  express more genes associated with effector function, proapoptotic signaling, and certain chemokines [103-105]. This correlates with the difference in effector function between  $T_{CM}$  and  $T_{EM}$ ; the former lack immediate effector function and are less differentiated, while the latter have immediate effector function and are further differentiated. A progressive differentiation pathway based on signal strength and/or extent of activation has been proposed, with naïve T cells as the least differentiated cells, followed by  $T_{SCM}$ ,  $T_{CM}$  and  $T_{EM}$  cells in a differentiation hierarchy (**Figure 3**) [42,43,106]. Together, these memory T cell subsets function as precursors for  $T_{EFF}$ .

Some studies indicate that arresting effector differentiation of antigen-specific CD8 T cells enables them to differentiate into memory T cells. For example, antagonizing IL-2 with IL-21 has been shown to increase the generation of  $T_{CM}$  [107,108] and induction of Wnt/ $\beta$ -catenin signaling using inhibitors of glycogen-synthase-kinase (GSK)-3 $\beta$  or Wnt3a protein induces the generation of  $T_{SCM}$  [42]. GSK-3 $\beta$  inhibition mimics Wnt signaling by promoting accumulation of  $\beta$ -catenin, the molecule that forms complex with Tcf1 and Lef transcription factors for regulating gene expression [42]. Tcf1 mediates signaling downstream of the Wnt pathway and promotes the development of memory T cells [42]. Mice lacking Tcf7 gene, which encodes Tcf, have a more differentiated effector/effector memory cell phenotype (i.e., CD44<sup>high</sup>CD62L<sup>low</sup>) [109,110].

The forkhead-box O (Foxo) family of transcription factors is a well-defined target of Akt. Akt phosphorylation at conserved sites of Foxo proteins triggers their nuclear exclusion and inactivation. Foxo1 and Foxo3 are the predominant Foxo members expressed within immune cells [111]. Foxo1, in particular, controls  $T_{CM}$  responses to infection [25] and is highly expressed in memory-precursor T cells. Foxo1 binds to and regulates expression of Tcf7 and Ccr7, which have critical functions in  $T_{CM}$  formation and trafficking. Deletion of Foxo1 causes defective secondary, but not primary, CD8 T cell responses to *Listeria monocytogenes* in mice [25]. Thus far, Foxo3 has no established role in mediating recall response of CD8+ T cells, as demonstrated by an antigen-specific *in vivo* study [112].

Id3 plays an important role in regulating the transition of activated CD8 T cells into effector cells and memory cells

[27,100,113]. Studies using mice expressing a reporter for Id3 have shown that Id3<sup>+</sup> memory precursors occur before the peak of T cell population expansion or upregulation of cell surface receptors associated with memory potential [27]. It is likely that Id3 is important for preserving proliferating CD8 T cells with memory potential early during priming and expansion phase. Loss of Id3 leads to defective formation of long-lived memory cells [27]. Ectopic expression of Id3 reportedly enhances recall response capability of tumor-reactive CD8 T cells and increases the production of memory precursor cells in mice [100]. High expression of Id3 preferentially guides the transition to memory cells, whereas low expression of Id3 leads to differentiation into effector cells [27].

Reducing the abundance of pro-differentiation transcription factors T-bet and Eomes may potentiate the generation of memory T cells. During acute response, CD8 T cells lacking both T-bet and Eomes lose CTL identity, and generate KLRG1<sup>low</sup> memory precursor cells, including both  $T_{SCM}$  and  $T_{CM}$ . However, their effector recall response capability is impaired upon reencounter of the antigen [114]. In addition, in memory CD8 T cells, Eomes sustains homeostatic survival and proliferation of memory cells through regulating IL-2R $\beta$  expression [26]. Loss of Eomes leads to decreased IL-2R $\beta$  expression, which is required for IL-15-mediated signaling and homeostatic proliferation of memory cells in the absence of antigen. Mice lacking Eomes reportedly have impaired turnover of long-term memory cells, largely due to reduction of IL-2R $\beta$  [26]. Furthermore, despite promoting the generation of memory T cells, reduction of Eomes and T-bet levels simultaneously leads to diminished effector capability. New approaches are needed to investigate if Eomes and T-bet might play an important role in regulating recall responsibility of memory T cells.

**Recall of effector functions.** It is noteworthy that the mechanisms for effector function recalled in memory cells differ from that of the primary effector response. For example, Id2 is required for the survival and expansion of effector cells generated during primary response, but is dispensable for reactivation of effector function by memory CD8 T cells [99]. Blimp-1-deficient effector CD8 T cells are reportedly generated and showed some reduction in expression of effector molecules [91-93]. Both  $T_{EFF}$  and  $T_{EM}$  have decreased proliferative capacity when rechallenged by their specific antigen. In contrast, loss of Blimp-1 leads to a faster development of  $T_{CM}$  and has no impact on recall response of memory T cells to become effector cells [92]. It is likely that other transcription factors are required for regulating the recall response capability of memory T cells. Alternatively, reactivation of effector function by memory cells may involve a multitude of mechanisms rather than a single transcription factor.

#### Interplay between Cytokines Signals and Transcription Factors in Memory Cells

Emerging evidence indicates that T cell heterogeneity is dictated during the antigenic priming phase and can be further modified in response to environmental stimuli. TCR ligation and inflammatory cytokines such as IL-12 and IFN- $\gamma$  upregulate T-bet in activated CD4 and CD8 T cells [26,88,115]. Some studies report that APC-derived Notch ligand activation of Notch signaling in T cells upregulates their expression of T-bet and Eomes and results in differentiation of effector T cells [19,21,23]. Notch signaling is also known to be important for induction of Gata3 and ROR $\gamma$ t in Th2 and Th17 cells, respectively [21,23,24,116,117]. Thus, both the degree and type of inflammatory stimulation serve to establish higher levels of lineage-specifying transcription factors (e.g., T-bet, Eomes, GATA3, ROR $\gamma$ t) and induce distinct lineages of effector cells [9].

Recent studies suggest that inflammatory cytokines regulate expression of Id2 and Id3 in activated CD8 T cells. Using Id2-YFP and Id3-GFP reporter mice, Goldrath and colleagues assessed the effect of cytokines on CD8 T cell expression of Id2 and Id3 during antigen-driven immune response [27]. While *in vitro* treatment with IL-2, IL-12 or IL-21 resulted in increase of Id2, *in vivo* experiments further confirmed the effect of IL-2 signaling on Id2 upregulation [27]. However, inactivation of IL-12 did not affect the expression of Id2. Thus, it is likely that IL-2 is a critical factor upregulating Id2 *in vivo*, whereas IL-12's effect may be redundant *in vivo* when IL-2 is available [27]. In contrast, IL-12 lowers Id3 expression in antigen-activated CD8 T cells in an *in vivo* experimental model, suggesting that IL-12 induction of effector differentiation leads to the down-regulation of Id3 [27]. The observation that IL-12 upregulates T-bet in activated T cells and the increasing effector pool [9] suggests that it may be useful to determine how cytokines and transcription factors act in concert to modulate the expression of Id2 and Id3 in T cells for effector differentiation and memory formation.

### T Cell Heterogeneity And Protective T Cell Immunity

To achieve efficient protective T cell immunity against infection and tumor cells, antigen-specific T cells are partitioned into subsets of memory T cells with distinct homing, self-renewal and effector recall potential. Adoptive cellular immunotherapy (ACT) is emerging as a potentially curative therapy for patients with advanced cancer. A major caveat of ACT is the observation that antigen-experienced T cells at distinct differentiation states may have different antitumor activity *in vivo* [42,102,118-120]. For example, as compared to T<sub>EM</sub>, T<sub>CM</sub> are less differentiated [2-4,6,8,121,122], have greater ability to proliferate and produce functional effector T cells [2-4,6,8,121,122], and show increased antitumor activity relative in many experimental studies [42,118-120,123,124]. Our recent studies [41] and others [42] have identified a population of antigen-experienced T<sub>SCM</sub> in mice [42]. As compared to T<sub>CM</sub>

and T<sub>EM</sub>, T<sub>SCM</sub> have a greater ability to inhibit tumor progression. T<sub>SCM</sub> have also been discovered in humans and have superior antitumor immunity in humanized mouse models [43]. Recent studies by a separate group further confirmed the potency of human T<sub>SCM</sub> against minor histocompatibility antigens (miHAs) in mediating potent antitumor activity in humanized mice [125]. Therefore, both T<sub>SCM</sub> and T<sub>CM</sub> serve as source for the total pool of memory cells and effector cells. They both have the high degree of cell plasticity and lowest degree of effector function, with T<sub>SCM</sub> exhibiting these characteristics more potently [43,106,120]. The development of novel approaches which activate memory cells and generate secondary effector cells may have significant implications in augmenting the efficacy of ACT.

The importance of T cell heterogeneity is reportedly important for T cell immunity against chronic infection [89]. Using both human and mouse chronic infection models, Wherry and colleagues have demonstrated that differential expression of T-bet and Eomes in distinct subsets of virus-specific CD8 T cells cooperatively maintain the pool of antiviral CD8 T cells during chronic viral infection [89]. During chronic infection phase, antiviral CD8 T cells expressing high levels of T-bet are slowly proliferating cells, but undergo rapid proliferation in response to the specific antigen and produce terminal progeny cells expressing high levels of Eomes. The absence of T-bet causes a shift toward Eomes-expressing terminal progeny cells and impedes the control chronic viral infection. Deletion of Eomes results in failure to control chronic infection due to the reduction of terminal effector cells [89]. Thus, both the T-bet-dependent and Eomes-dependent subsets of antiviral CD8 T cells cooperatively contribute to an effective protective immunity against chronic infection.

CD4 T cells also provide effective protection against tumor and chronic infection. Recent studies suggest that CD4 T cells not only promote CD8 T cell function, but also play a direct role in tumor elimination [126-130]. The manner in which CD4 T cells mediate anti-tumor immune response depend on the generation of both IFN- $\gamma$ -producing progeny and cytolytic effector cells that can destroy tumor cells [127,128]. Notably, recent evidence suggests that CD4 Th17 cells help CD8 T cells to mediate long-term anti-tumor immunity [131,132]. Thus, efficient protection immunity against tumor and pathogen reflects collective efforts of differential subsets of antigen-specific T cells.

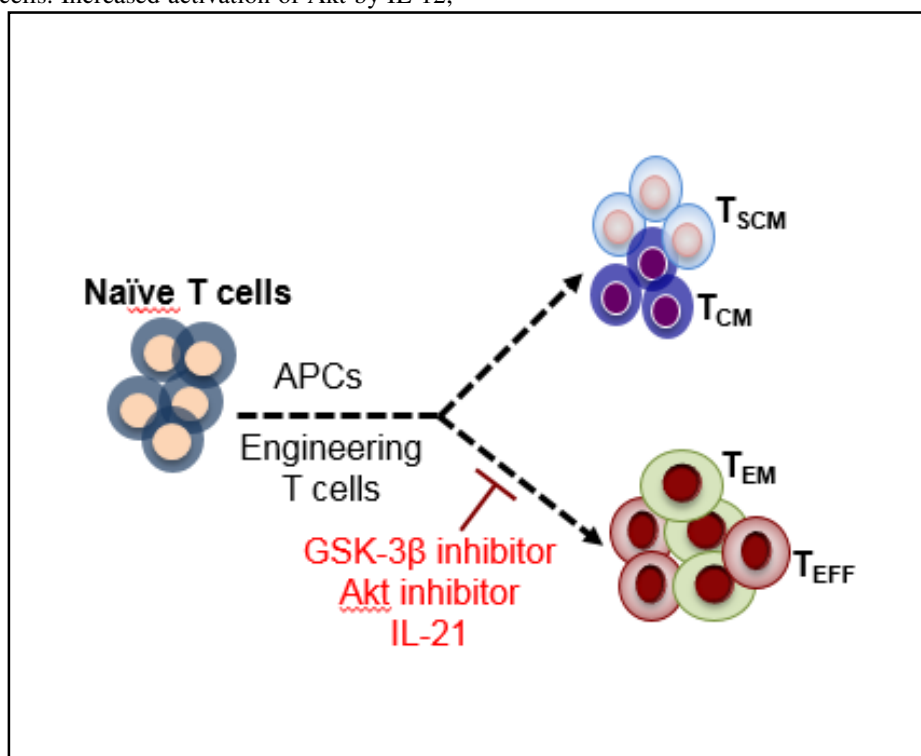
### Modifying T Cell Heterogeneity for Tumor Immunotherapy

One of the main barriers to improving the efficacy of ACT is ensuring the preservation of T cell self-renewal, which ensures the continuous production of progeny capable of eradicating tumor after adoptive transfer into patients [42,43,106,120]. Considerable efforts have been made to improve methods used for *ex vivo* expansion of tumor-

reactive T cells for ACT. An approach under active evaluation involves the growth of cells under conditions that enable *ex vivo* proliferation while limiting differentiation (Figure 4). The addition of GSK3- $\beta$  inhibitors into cultures has been shown to reduce effector differentiation and increase the frequency of both T<sub>SCM</sub> and T<sub>CM</sub> [42,43]. This subset of T<sub>SCM</sub> has greater ability than other subsets of memory T cells to control the growth of established tumors upon adoptive transfer [42,43].

TCR, IL-2 receptor and IL-12 receptor signaling have all been demonstrated to stimulate the PI3K/Akt signal transduction pathway [133-135]. Several studies suggest that PI3K/Akt is critical for proliferation and differentiation of activated CD8 T cells. Increased activation of Akt by IL-12,

expression of a constitutively active form of Akt and deletion of Foxo1, have all been shown to promote the formation of KLRG1<sup>hi</sup> effector cells [5,9,136]. A recent study shows that inhibiting the Akt pathway leads to generation of highly potent miHA-specific CD8 T cells *ex vivo* [125]. These Akt-inhibited CD8 T cells showed superior expansion potential upon removal of the Akt inhibitor, which results in a superior antitumor effect in a humanized mouse model [125]. Akt inhibition can also enhance persistence of tumor-infiltrating lymphocytes after adoptive transfer into an immunodeficient animal model and augment antitumor immunity of CD8 T cells [137].



**Figure 4. Arresting CD8 T Cell Differentiation May Improve ACT Efficacy.** ACT is limited by the capacity of transplanted cells to provide continual supply of functional effector cells while retaining self-renewal. An area of active investigation involves the development of an *ex vivo* culture system that promotes cell proliferation while limiting differentiation. GSK3- $\beta$  inhibitors employed in *ex vivo* cultures have been shown to reduce effector differentiation and increase the frequency of both T<sub>SCM</sub> and T<sub>CM</sub> [43]. Akt inhibition has been demonstrated to enhance proliferation [125] and persistence [137] of anti-tumor immune cells. Some studies suggest that addition of IL-21 has the potential to arrest differentiation without affecting proliferation [108,140].

Cytokines such as IL-15 and IL-21 can sustain T-cell proliferation while limiting excessive differentiation, exhaustion, and senescence. T cells cultured in IL-15 display a T<sub>CM</sub>-like phenotype and gene expression profile, and have greater anti-tumor function in mice than T cells cultured in IL-2 [138,139]. IL-21 modulates the differentiation of activated T cells and results in development of a population of cells characterized by a T<sub>SCM</sub> phenotype [108,140]. Human T cells cultured in IL-21 retain the ability to release

IL-2 and express markers associated with a minimal differentiated phenotype (e.g., CD45RA, CD28, CD27, IL7Ra and CD62L) [108,140,141]. In a mouse model of melanoma, T cells derived from IL-21 cultures demonstrated markedly enhanced anti-tumor activity compared with cells grown in the presence of other cytokines [108].

The CD27-dependent pathway of T-cell expansion has therapeutic potential to enhance the efficacy of ACT. CD27



is highly expressed on the surface of naïve CD8 T cells [142-145]. Activating CD27 by soluble CD70 promotes cellular expansion of CD8 T cells in the absence of IL-2 without causing significant effector differentiation [142,143,145]. This effect of CD27 signaling resulted in increased cell cycling and survival that was mediated in part by upregulation of IL-7Ra on the T cell surface [142,143,145]. Data from animal experiments also indicate that CD27-null CD8 T cells have impaired primary and secondary expansion in mice challenged by influenza and poliovirus. Finally, CD27 is reported to mediate the generation of antigen-experienced CD8 T cells with memory traits [142,145,146]. Further preclinical studies using ACT models are necessary to evaluate the validity of CD27-dependent expansion of T cells as a feasible approach to improve the efficacy of ACT for patients with advanced cancer.

### CONCLUDING REMARKS

This review has highlighted the significant progress that has been made in understanding how transcription factors regulate the development of T cell heterogeneity. A multitude of transcription factors coordinate their activities to orchestrate distinct transcriptional programs that direct the differentiation and maintenance of a functionally diverse group of T cell subsets. The upstream molecular pathway(s) involved in orchestrating the expression of these subset-specific transcriptional programs remain a critical unresolved question. The continued exploration of transcriptional control of T cell heterogeneity will have broad implications in identifying novel pathways that may be targeted to create therapies for autoimmune diseases, chronic infections and complications involved with transplantation, including graft rejection and GVHD.

In addition, a greater understanding of transcriptional programs controlling terminal differentiation and memory formation will have an immediate impact on T cell-based anti-tumor therapies such as ACT. During ACT, a strong antitumor effect in patients with advanced cancer can be achieved by transfer of large amount of cytolytic effector T cells. Current *in vitro* methods used to expand tumor-reactive T cells are ineffective in maintaining a population of minimally differentiated T cells while generating sufficient cell numbers. The predominant obstacle to retaining this population is the fundamental coupling of clonal expansion and effector differentiation. This coupled expansion and differentiation impairs the generation of memory T cells that are able to persist and replicate to elaborate effector function for eliminating tumor *in vivo* following adoptive transfer. Further exploration of the molecular mechanisms whereby T cells closely link expansion and differentiation will lead to new strategies to improve the efficacy of cancer immunotherapy.

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