Review Article

Functional and Phenotypic Plasticity of CD4$^+$ T Cell Subsets

Tiffany Caza and Steve Landas

Department of Pathology, Upstate Medical University, 750 East Adams Street, Syracuse, NY 13210, USA

Correspondence should be addressed to Tiffany Caza; cazat@upstate.edu

The remarkable plasticity of CD4$^+$ T cells allows individuals to respond to environmental stimuli in a context-dependent manner. A balance of CD4$^+$ T cell subsets is critical to mount responses against pathogen challenges to prevent inappropriate activation, to maintain tolerance, and to participate in antitumor immune responses. Specification of subsets is a process beginning in intrathymic development and continuing within the circulation. It is highly flexible to adapt to differences in nutrient availability and the tissue microenvironment. CD4$^+$ T cell subsets have significant cross talk, with the ability to "dedifferentiate" given appropriate environmental signals. This ability is dependent on the metabolic status of the cell, with mTOR acting as the rheostat. Autoimmune and antitumor immune responses are regulated by the balance between regulatory T cells and Th$_{17}$ cells. When a homeostatic balance of subsets is not maintained, immunopathology can result. CD4$^+$ T cells carry complex roles within tumor microenvironments, with context-dependent immune responses influenced by oncogenic drivers and the presence of inflammation. Here, we examine the signals involved in CD4$^+$ T cell specification towards each subset, interconnectedness of cytokine networks, impact of mTOR signaling, and cellular metabolism in lineage specification and provide a supplement describing techniques to study these processes.

1. An Introduction to CD4$^+$ T Cell Diversity

Production of a diverse repertoire of antigen-specific CD4$^+$ T lymphocytes is essential for a host to respond to emerging microbial threats to create memory for heightened secondary responses to previously encountered pathogens and to suppress immune responses after microbial clearance to avoid tissue damage resulting from excessive or protracted inflammation [1]. Plasticity of CD4$^+$ T cells is required to maintain immunocompetence after the thymic involution in adulthood [2]. Varying functional CD4$^+$ T cell clones are also required to operate immune responses in different tissues as well as to produce high-affinity, class-switched immunoglobulin [3].

It is hypothesized that CD4$^+$ T cells undergo subset specification but not lineage determination [3]. CD4$^+$ T cells mature to form subsets with specified phenotypes and differences in cytokine production but fall short of terminal differentiation. Specification is a reversible maturation process that allows CD4$^+$ T cells to undergo alternate fates, depending on environmental signals received. Signals contributing to subset specification include the prevailing cytokine environment, cytokine receptor expression profiles, transcription factor expression, and differential chromatin remodeling of loci that regulate production of effector cytokines [4].

Naïve CD4$^+$ T cells undergo specification by many innate immune signals, including cytokines, chemokines, and inflammasome activation, which result in activation of signal transducers and activators of transcription, subsequent activation of lineage-specific transcription factors, cytokine production, and epigenetic adjustments at the cytokine loci to result in commitment to a given lineage.

Once a naïve T cell is primed by signals received from an antigen-presenting cell, proliferation occurs before lineage specification begins. If differentiation of CD4$^+$ T cells occurred early after priming, peripheral CD4$^+$ T cells would be restricted with binary options, being able to turn on or repress production of only a restricted subset of cytokines [5]. Subset determination occurring after clonal proliferation is consistent with an activated CD4$^+$ naïve T cell producing

many diverse progeny with pleiotropic, distinct fates, producing a highly flexible, dynamic, and context-driven CD4^{+} T cell repertoire [5].

Surprisingly, CD4^{+} T cell that has undergone lineage specification is capable of adopting alternate fates when innate immune signals change. The molecular basis for cytokine memory involves imprinting gene loci encoding cytokines by demethylation of DNA or histone acetylation as cells progress through S phase, so stable patterns of gene expression occur with an increasing number of cell divisions [6]. Yet, later chromatin remodeling occurs within CD4^{+} T cells to turn on new cytokine production profiles [5].

In this review, we will first examine functional differences between CD4^{+} T cell subsets and their lineage specification. A focus on the interconnectedness among pathways of maturation will follow with a presentation of experimental evidence supporting the hypothesis that CD4^{+} T cells maintain plasticity. The role of mTOR and cellular metabolism in T cell differentiation and function will be discussed. Finally, the impact of CD4^{+} T cell subsets in immunopathology and in antitumor immune responses will be considered.

2. T Cell Subsets and Lineage Specification

2.1. CD4^{+} T Cell Diversity Begins during Development. Diversity of the CD4^{+} T cell repertoire begins during intrathymic development. Thymocyte differentiation produces a diversity of CD4^{+} T cells with varying antigen specificities through β-selection, followed by α-chain rearrangement to form diverse $\alpha\beta$ TCR specificities [27]. CD4 lineage selection is mediated through interaction of the T cell receptor (TCR) with class II MHC ligands. CD4^{+} T cell development is promoted by high TCR signal strength and signaling downstream of the TCR contributes to CD4 lineage commitment through association of Lck with the CD4 coreceptor and MAP kinase signaling to favor maintenance of CD4 expression with concurrent downregulation of CD8 [28]. CD4 commitment is mediated through induction of the transcription factor, T helper-inducing POZ/Kruppel-like factor (Th-POK), by GATA3 which represses Runx3 to release activity of the CD4 silencer [10, 29].

The lineage decision of commitment to the CD4^{+} or CD8^{+} T cell lineage was thought to be committed and inflexible, although it is now understood that there is a high degree of latitude in the CD4^{+} T cell compartment [3]. Lineage commitment is regulated not only through positive and negative selection but also through additional mechanisms. Helper-deficient (HD) mice, which lack the ability to produce CD4^{+} T cells, have spontaneous redirection of MHC class II-restricted T cells to the CD8^{+} lineage. The factor identified to be responsible for the redirection of the MHCII-restricted T cells to the CD8^{+} lineage was a mutation within the transcription factor, ThPOK. Wild-type ThPOK suppresses the cytolytic gene expression profiles in CD4^{+} T cells to induce lineage maturation and is both required and sufficient for maturation of the CD4^{+} T cell lineage. It was recently identified that antigen-experienced CD4^{+} T cells can turn off ThPOK to reactivate genes of the CD8

lineage, showing that this maturation step in intrathymic development is not fixed [30].

Early after priming by the innate immune response, CD4^{+} T cells are able to undergo conversion to an alternate subset through cytokine and chemokine receptor signaling, which induces changes in transcription factor expression [3]. T cell subset specification is influenced by interactions with dendritic cells (DCs) or peritoneal macrophages, the dose and form of presented antigen, the affinity of peptide-TCR interaction, cytokines, and costimulatory interactions [4, 31]. Asymmetric cell division during the DP to SP transition in intrathymic development also influences CD4^{+} T cell lineage decisions as daughter cells may "inherit" unequal shares of signaling molecules due to altered positioning across autocrine or paracrine chemokine gradients, influenced in part by Notch signaling [10]. Notch binding DLL 1 and 4 ligands promote lineage commitment to the Th_1 subset, while Notch-Jagged interactions result in Th_2 specification [7].

During at least the first several rounds of cell division under polarizing conditions, Th subset populations are heterogeneous, have low frequencies of cytokine producing cells, and have reversible phenotypes and effector cytokine production [4]. CD4^{+} T cells are plastic at this stage and beyond and are capable of switching their phenotypes to produce cytokines based on their activation status, environment, and metabolism. Reversibility is possible because the lineage-specific transcription factors that act as master regulators for subset specification are not fully repressed in other lineages but carry both permissive and repressive epigenetic marks or bivalency [3]. Bivalent epigenetic marks allow for rapid transition between active transcription and repressed transcription [32]. CD4^{+} T cells maintain flexibility in expression of genes encoding transcription factors that regulate cytokine loci, allowing adaptation to altered programs of cytokine expression in a potentially damaging inflammatory milieu [12].

2.2. CD4^{+} T Cell Subsets. Identification and characterization of CD4^{+} T cell lineage subsets began nearly three decades ago with the landmark papers of Mosmann et al., which described and identified two CD4^{+} T cell subsets, Th_1 and Th_2 [8, 33–35]. Subsets of CD4^{+} T cells were identified based on production of cytokines in specific responses to antigen or generalized stimulation with Con A [33, 34]. It was identified that Th_1 cells produce IL-2 and IFNγ, while Th_2 cells produced IL-4, IL-5, preproenkephalin, and p600 [34]. Both clones produced IL-3, GM-CSF, and TNF. They further defined the role of Th_1 cells in mediating antigen-specific, MHC restricted, delayed type hypersensitivity reactions for a variety of antigens, while this ability was absent in Th_2 cells [8]. Additionally, it was shown that Th_2 cells produce a "cytokine synthesis inhibitory factor" capable of inhibiting Th_1 cytokine production without a change in viability of the Th_1 clones [35]. These discoveries first identified that CD4^{+} T cells were functionally and phenotypically heterogeneous and capable of cross talk.

One mechanism in which CD4^{+} T cells undergo subset specialization is through responding to cytokine signals produced in the innate immune response, inducing activation

of lineage-specific transcription factors that result in production of a set of effector cytokines [4]. The initial priming cytokines are those produced by antigen presenting cells (APCs) [5]. Activated APCs deliver three types of signals required for the clonal expansion and maturation of CD4$^+$ T cells [31]. The first signal is mediated by the peptide-MHC interaction with the TCR. The second involves costimulatory interactions between the APC and the T cell. The third signal directs differentiation of naïve T cells to effector T cell subsets through cytokines, Ras-MAPK signaling, and Notch ligand interactions [31, 36]. Pathogen recognition by macrophages and dendritic cells of the innate immune response initiates a signaling program that stimulates T lymphocytes and initiates adaptive responses [37]. The fate of a naïve T cell to undergo subset differentiation depends upon cytokine signaling and activation of proteins of the signal transducer and activation of transcription family (STATs). STAT activation is mediated by Janus kinases (JAKs) that are induced during the initial priming period. JAK-STAT triggering leads to activation of lineage-specific transcription factors, which results in expression of effector cytokines [4].

The differential function of CD4$^+$ T cells is determined through which specific cytokines they produce [38]. Cytokines responsible for induction of CD4$^+$ T cell differentiation, lineage specific transcription factors activated during subset specification, effector cytokines produced, and general functions of T cell subsets are summarized in Table 1.

Known CD4$^+$ T cell subsets include Th$_1$, Th$_2$, Th$_{17}$, Th$_9$, Th$_{25}$, T follicular helper cells (T$_{FH}$), and regulatory T cells (T$_{reg}$). Th$_1$ cells are produced in response to intracellular pathogens (including parasites, viruses, and intracellular bacteria) and mediate cell-mediated immunity and delayed-type hypersensitivity reactions [8]. The Th$_1$ program is induced by IFNγ produced by natural killer (NK) and dendritic cells, which activates STAT1, resulting in activation of the lineage-specific transcription factor T-bet [9]. IL-27, a cytokine of the IL-12 family, also contributes to STAT1 phosphorylation and T-bet activation. T-bet expression increases production of the IL-12 receptor, which activates STAT4, leading to activation of IFNγ transcription and subsequent IFNγ production [7]. This serves as positive feedback, stimulating more naïve T cell clones to undergo Th$_1$ specification to polarize the immune response towards fighting an intracellular pathogen. Th$_1$ cells also produce TNFα and lymphotoxin, cytokines which trigger neutrophil chemotaxis and macrophage activation to potentiate innate immune reactions [38, 39]. Th$_1$ cells also help B cells in antibody class-switching to produce high-affinity IgG for opsonization of an offending pathogen [40].

Th$_2$ specification is required for B cell help in humoral immunity and elimination of extracellular microbes and intestinal helminthes [5, 40]. Th$_2$ cells are involved in antibody class-switching to produce IgE which can provoke or sustain allergic reactions [8, 36, 39]. Differentiation of the Th$_2$ subset requires IL-4 produced by Notch ligand activation of dendritic cells which, in turn, induces STAT6, which activates the lineage-specifying transcription factor, GATA-3 [36]. GATA-3 activates transcription of the Th$_2$ cytokine cluster leading to IL-4, IL-5, and IL-13 production. Th$_2$ cytokines provide positive feedback for maturation of naïve

T cells to the Th$_2$ lineage and inhibit Th$_1$ development [39] by the homeostatic cytokine IL-10. Th$_2$ cells also heighten the innate immune response through activation of macrophages by induction of IL-4 and macrophage activating factor (MAF) [5].

Th$_{17}$ cells provide protection against bacteria and fungi at mucosal surfaces and confer coverage of some microbes that are not targeted in Th$_1$ or Th$_2$ responses, including, but not limited to, *Mycobacterium tuberculosis*, *Bacteroides fragilis*, and *Klebsiella pneumoniae* [15]. Induction of the Th$_{17}$ lineage occurs when IL-6, IL-23, and TGFβ are present in the inflammatory milieu without IL-4 or IL-12 (which promote Th$_2$ or Th$_1$ responses, resp.) [41, 42]. Toll-like receptor signaling, leading to MyD88 signaling, is another innate immune signal fostering Th$_{17}$ differentiation [43]. IL-6 promotes STAT3, which induces retinoic orphan receptor (ROR) transcription factors, RORα and RORγT, leading to production of Th$_{17}$ cytokines IL-17, IL-17F, and IL-22 [44, 45].

Mucosal immunity is provided through Th$_9$, Th$_{22}$, and IL-25 producing cells [46]. Th$_9$ cells provide protection against intestinal helminth infections [13]. IL-9 producing cells are proinflammatory as they stimulate proliferation and inhibit apoptosis of hematopoietic cells and also activate Th$_{17}$ cells [47]. This is due to stimulation of Jak1 by IL-9, resulting in activation of STATs 1, 3, and 5. Th$_9$ cells undergo a maturation program similar to Th$_2$ cells, with IL-4 inducing STAT6 activation and produce the Th$_2$ cytokines IL-9 and IL-10, but, unlike Th$_2$ cells, they require TGFβ for maturation [13, 48]. The lineage-specific transcription factor for Th$_9$ development may be the activator protein 1 family transcription factor, BATF, leading to a transcriptional program which results in increased IL-9 and IL-10 production [13, 49]. Th$_{22}$ cells are CD4$^+$ T cells that are phenotypically and functionally related to Th$_{17}$ cells that participate in wound repair and in protection against bacterial, viral, and fungal infections at epithelial surfaces, including the skin and GI tract [21]. They prevent translocation of microbes across epithelia, which limit the extent of infection [18]. Th$_{22}$ specification is promoted by IL-6 and TNF-α, which induces STAT3, and expression of the aryl hydrocarbon receptor [19]. This parallels Th$_{17}$ maturation, and numerous phenotypic markers are expressed in common between Th$_{17}$ and Th$_{22}$ cells, including CCR6, CCR4, dipeptidyl peptidase IV, CD26, and CD90 [20]. CCR10 is also expressed on Th$_{22}$ cells, distinct from Th$_{17}$ [20]. Th$_{22}$ cells produce IL-22, IL-13, fibroblast growth factor, CCL15, CCL17, and TNFα at epithelial surfaces. IL-22, an IL-10 family cytokine, production is not unique to Th22 cells but is also produced by Th$_1$ and Th$_{17}$ cells; however, Th$_{22}$ cells can produce IL-22 in the absence of IFNγ or IL-17 [20]. IL-25-producing cells may represent a new subset, Th$_{25}$ cells, which stimulate nonlymphoid cells to produce effector cytokines in response to extracellular pathogens [22]. They are induced by the transcription factor Act1, but can be derived from the Th$_2$ lineage [12, 46]. IL-25-producing cells and the Th$_2$ subset may be linked as IL-4 is required for production of both cell types and IL-25 enhances production of Th$_2$ cytokines, inducing IL-4, IL-5, and IL-13 secretion [12, 50].

TABLE 1: Characterization of CD4+ T cell subsets.

Th subset	Factors inducing lineage	STAT activated	Lineage-specifying transcription factor	Effector cytokines produced	Functions
Th$_1$	IL-12 IL-27	STAT4, STAT1	T-bet	IFNγ, lymphotoxin, TNFα	Cell-mediated immunity, delayed-type hypersensitivity responses, clearance of intracellular pathogens and tumor cells, opsonizing Ab production by B cell class-switching to IgG2a [3, 4, 7–9]
Th$_2$	IL-4 Indoleamine 2,3-dioxygenase	STAT6	Gata-3 c-MAF	IL-4, IL-5, IL-13, IL-10	Humoral immunity, clearance of extracellular bacteria and worms, B cell class-switching to IgE, allergic responses [3, 4, 7, 10, 11]
Th$_9$	IL-4 TGFβ	STAT6	BATF	IL-9, IL-10	Protection against parasitic worms/helminth infections [12, 13]
Th$_{17}$	IL-6 MyD88 Low TGFβ IL-23	STAT3	RORγT, RORα	IL-17, IL-17F, IL-6, IL-22, TNFα, IL-10	Protection of mucosal surfaces, recruitment of neutrophils, clearance of *Mycobacterium tuberculosis* and *Klebsiella pneumonia* [3, 14–17]
Th$_{22}$	IL-10Rβ IL-6 TNFα	STAT3	Aryl hydrocarbon receptor	IL-22, IL-13, FGF, CCL15, CCL17, TNFα	Mucosal immunity; prevention of microbial translocation across epithelial surfaces, promotes wound repair. [18–21]
Th$_{25}$	IL-4, IL-25	Unknown	Actl	IL-25, IL-4, IL-5, IL-13	Mucosal immunity, stimulates nonlymphoid cells to produce IL-4, limits Th$_1$ and Th$_{17}$ induced inflammation, CD4+ T cell memory (mouse) [12, 22]
T$_{FH}$	Strong TCR signal, IL-12, CXCR5, IL-21, IL-4	STAT3	MAF (IL-21 transactivator)	IL-21, OX40, ICOS	Helps B cells produce high affinity, class-switched antibodies, guides migration into germinal centers [23, 24]
T$_{reg}$ (includes Tr1 and Th$_3$ cells)	High TGFβ mTOR	STAT5	Foxp3	IL-10, TGFβ	Suppression of existing immune responses, maintains tolerance/protection against autoimmunity [15, 16, 25, 26]

T follicular helper cells (T_{FH}) improve B cell class-switching for immunoglobulin production and guide B cells into germinal centers by chemotaxis mediated by CXCR5 signaling [23]. T_{FH} cells require a strong TCR signal for induction, which is also required for T_{reg} responses [24, 51]. T_{FH} specification requires activation of the inducible costimulator (ICOS), a CD28-related costimulatory signal provided by activated dendritic cells or B cells, which initiates transcription of the transcription factor MAF that transactivates IL-21 [24]. OX-40/CD134 ligation is another required costimulatory signal, which downregulates CTLA-4, a dominant suppressor of T cell activation [24]. IL-6 and STAT3 are required for T_{FH} development similar to Th_{17} cells, yet T_{FH} cells can be generated in the absence of Th_{17} cytokines, IL-17, IL-17F, or TGFβ [23].

Suppression of immune responses and maintenance of peripheral tolerance is provided by T_{reg}s [25]. T_{reg}s are a heterogeneous population which includes thymic-derived natural T_{reg}s (nT_{reg}s), adaptive regulatory T cells involved in maintaining oral tolerance (Th_3 cells), and T regulatory type 1 cells (Tr1 cells), induced by IFNα secreted by neighboring plasmacytoid dendritic cells (pDCs). nT_{reg}s require a strong TCR signal, which is potentially self-reactive, for development [52]. They are generated with minimal costimulation, for T cell recognition of antigen without a strong second signal from a CD28 family member can provide induction of tolerance [52]. Differentiation of induced T_{reg}s, Th_3 cells, and Tr1 cells occurs in the periphery and requires high concentrations of TGFβ, with the absence of proinflammatory cytokines [15]. Cell-cell contact and IL-10 secretion is required for suppressor function, mediated through STAT5-induced activation of the lineage-specific transcription factor Foxp3, with concurrent downregulation of the Th_{17} transcription factor RORγT [16]. Suppressor function of T_{reg}s requires Foxp3 expression [53]. Reduced T_{reg} numbers and effector function occur in autoimmune diseases and complete deficiency of this subset results in a severe autoimmune disease, immune dysregulation, polyendocrinopathy, and enteropathy with X-linked inheritance (IPEX) syndrome [52].

A population of non-T_{reg} Foxp3-expressing CD4$^+$ T cells has been identified, which is known as the "exFoxp3" T cell [54]. exFoxp3 cells have transient Foxp3 expression in an activated state, and these cells can accumulate at sites of inflammation. These represent effector T cells that gain Foxp3 expression and not conversion to the T_{reg} lineage. A small population of T_{reg}s with loss of Foxp3 expression while maintaining commitment to the T_{reg} lineage also exists. This is through demethylation at the TSDR locus, which retains memory of its suppressor phenotype [54].

Central memory CD4$^+$ T cells, created through initial priming and restimulation, consist of a heterogeneous population that is not lineage-committed, for memory responses are subject to manipulation under cytokine-polarizing conditions to adapt to new antigenic stimuli [55]. Effector memory CD4$^+$ T cells are thought to have undergone lineage determination. Plasticity of the central memory population is essential for maintenance of specific CD4$^+$ T cells after pathogen clearance, since 90–99 percent of Th_1 or Th_2 effector cells will undergo apoptosis after antigenic challenge [56].

3. Cross Talk and Flexibility in T Cell Subset Lineage Specification

Effector cytokines produced by CD4$^+$ T cells provide positive feedback to increase further differentiation of naïve T cells to that lineage while inhibiting differentiation of opposing subsets [57]. Signal transduction pathways induced by cytokines and chemokines influence lineage commitment events through activation or repression of a subset-specific transcriptional program [58].

Th_1 *and* Th_2 *Subsets.* Commitment to the Th_1 lineage inhibits Th_2 development, and Th_2 commitment inhibits Th_1 responses [39]. IFNγ production by Th_1 cells inhibits production of Th_2 cytokines [39]. Likewise, IL-4 produced during Th_2 specification inhibits production of IFNγ and IL-12, preventing differentiation of naïve CD4$^+$ T cells to the Th_1 lineage [39]. GATA3 expression by Th_2 cells leads to upregulation of sphingosine kinase I expression and downregulation of STAT4, which inhibit Th_1 development [59].

Plasticity occurs between the Th_1 and Th_2 lineages, and early after naïve CD4$^+$ T cell activation, production of IFNγ and IL-4 can occur simultaneously [55]. Decreased expression of intracellular osteopontin by APCs with increased soluble osteopontin produced by T cells (increased soluble-to-intracellular osteopontin ratio) stimulates IL-12 production to promote Th_1 lineage commitment [60]. With culture of CD4$^+$ T cells in a Th_1-promoting environment (containing IL-12 and anti-IL-4 antibody), the population of cells will polarize to produce IFNγ. Removal of the polarized CD4$^+$ T cells into IL-4 containing medium promotes Th_2 cytokine production, displaying the capacity of converting between the two phenotypes. In addition, forced overexpression of GATA3 in Th_1 polarized cells results in conversion to a Th_2 phenotype, while T-bet overexpression in Th_2 polarized cells results in a Th_1 phenotype [61]. Flexibility between Th_1 and Th_2 cytokine production is lost, however, with repeated stimulation and multiple rounds of cell division [6]. This is thought to be due to chromatin remodeling at cytokine loci to increase efficiency of effector cytokine production and inhibit opposing cytokine programs [62].

Intrachromosomal interactions through modifications of chromatin structure are also responsible for repression of the alternate lineage program [32]. Stimulation under Th_1 or Th_2 polarizing conditions results in altered chromatin accessibility after 4 to 6 cell divisions. In naïve T cells, the IFNγ locus is bivalent, poised for enhancing gene expression or transcriptional silencing, depending on which signals are received. Under a Th_2 polarizing cytokine environment, permissive histone modifications are lost at the IFNγ locus by DNA methylation. Similarly, repressive methylation at the Th_2 locus occurs during Th_1 polarization [63]. In addition to intrachromosomal modifications, interchromosomal interactions exist between the IFNγ and Th_2 cytokine clusters for negative regulation of the opposite lineage. Direct interaction

between the IFNγ promoter and regulatory regions of the Th$_2$ cytokine cluster cross-regulate one another [64]. Naïve T cells have the ability to express both Th$_1$ and Th$_2$ cytokines within hours of T cell activation due to the interaction of these two loci creating a chromatin hub configuration between the IFNγ promoter and the Th$_2$ locus control region [64].

Expressions of Th$_1$ and Th$_2$ cytokines from a single cell, as well as environments rich in both Th$_1$ and Th$_2$ cytokines, further show flexibility in subset specification. When CD4$^+$ T cells are stimulated *in vitro* with IL-12, they produce both IFNγ and IL-4 [65]. Yet, repeated stimulation will reduce the percentage of cells expressing this phenotype, suggesting that the double-positive cells represent a transition state of differentiation.

In vivo polarization experiments using model pathogens have also demonstrated interconversion between Th$_1$ and Th$_2$ cells. CD4$^+$ T cells exposed to *Leishmania major* infection differentiate into the Th$_1$ lineage and produce IFNγ while maintaining the capacity of interchanging into a Th$_2$ phenotype when exposed to IL-2 and IL-4 *ex vivo* [65]. There is the possibility, however, that reversal from a Th$_1$ to Th$_2$ phenotype may simply reflect the outgrowth of a population of uncommitted cells rather than dedifferentiation from a Th subset [65].

T$_{reg}$ and Th$_{17}$ Subsets. Th$_{17}$ and T$_{reg}$ subsets are in a homeostatic balance and are derived from a common precursor. CD4$^+$ T cells with dual expression of Foxp3 and RORγT exist during early Th17 cell development and in naïve T cells after stimulation with TGFβ. These Foxp3$^+$RORγT$^+$ expressing cells may occur as an intermediate during commitment to an effector lineage, T$_{reg}$ or Th$_{17}$ cells [66]. They mature into either a T$_{reg}$ or Th$_{17}$ cell depending on the cytokine profile in the environment [16]. IL-6, IL-21, IL-23, and low levels of TGFβ support induction of RORγT and Th$_{17}$ development. High levels of TGFβ, retinoic acid, and IL-2 support T$_{reg}$ commitment through activation of Foxp3 [15, 66]. Th$_{17}$ cells can be converted from induced T$_{reg}$ cell populations in the presence of the cytokines IL-6 and IL-1. When TGFβ expression is high and IL-6 is present, a population of Foxp3$^+$IL-17$^+$ cells results. STAT3 phosphorylation in cells committing to a Th$_{17}$ lineage inhibits TGFβ-induced Foxp3 expression [32]. Additionally, RORγT directly interacts with exon 2 of the Foxp3 gene to suppress T$_{reg}$ development and activates transcription of Th$_{17}$ cytokines. Similarly, Foxp3 can bind RORγT to suppress IL-17 production [66]. Other coexpressed transcription factors influence the T$_{reg}$ v. Th$_{17}$ lineage branch point. These include interferon regulatory factor-4 and Runx1, which promote Th$_{17}$ differentiation through interaction with the Foxp3 locus [29]. Both thymic-derived and peripherally induced T$_{reg}$s express Helios [67, 68], which could be used to identify whether a Th$_{17}$ cell was derived from a T$_{reg}$ versus being induced from a naïve T cell precursor. Low levels of Helios expression can indicate T cell activation, while high expression suggests T$_{reg}$ origin [69]. Proinflammatory cytokine production by Th$_{17}$ cells inhibits generation of T$_{reg}$ cells, and T$_{reg}$ production of IL-10 suppresses Th$_1$ and Th$_{17}$ generation [14, 25]. T$_{reg}$s antagonize Th$_{17}$ function and reduce IL-17 production when it is no

longer required for pathogen clearance to avoid tissue injury [16].

T$_{reg}$ and Th$_{17}$ cells are the predominant CD4$^+$ T cells within tumor microenvironments [70]. T$_{reg}$s suppress anti-tumor immune responses through promoting tolerance to the tumor by IL-10 production. In the presence of IL-6, TGFβ, type I interferons, IL-12, and intact toll-like receptor signaling via MyD88, Th$_{17}$ cell specification is induced from T$_{reg}$ cells [70, 71]. Overall, the number of T$_{reg}$s is increased in many cancers and has been shown in gastric adenocarcinoma [72], esophageal adenocarcinoma [72], squamous cell carcinoma (head and neck) [73–75], breast carcinoma [76], and non-small cell lung carcinoma [76]. Reduced numbers of intratumoral Th$_{17}$ cells have been associated with poor prognosis in several tumor models [70]. Yet, in a recent lung adenocarcinoma model, K-ras^{G12D} expression promoted recruitment of Th$_{17}$ cells to the tumor and increased tumor growth, with IL-17 blockade reducing tumor burden [77]. Whether an excess of T$_{reg}$s or Th$_{17}$ cells is pathogenic within a tumor could be context-dependent, based on the type of tumor, its oncogenic drivers, the microenvironment, and the immunocompetence (versus compromise) of the host.

Proinflammatory cytokine production by Th$_{17}$ cells inhibits generation of T$_{reg}$ cells, and T$_{reg}$ production of IL-10 suppresses Th$_1$ and Th$_{17}$ generation [14, 25]. T$_{reg}$s antagonize Th$_{17}$ function and reduce IL-17 production when it is no longer required for pathogen clearance to avoid tissue injury [16]. The T$_{reg}$ lineage may not be fixed, as T$_{reg}$s have been identified to differentiate into Th$_{17}$ or T$_{FH}$ cells. Foxp3$^+$ T cells in the thymus develop into Th$_{17}$ cells and produce IL-17 when taken *ex vivo* and put into IL-6-containing medium. Additionally, over one-fourth of Th$_{17}$ cells along the small intestine mucosa are thought to be derived from Foxp3$^+$ iT$_{reg}$s [15]. However, it is possible that differentiation into effector CD4$^+$ T cell subsets could represent a population of activated T cells with aberrant Foxp3 expression (exFoxp3 cells) rather than T$_{reg}$s themselves.

Whether plasticity of T$_{reg}$s exists is under debate; however, there is known heterogeneity within T$_{reg}$ populations. Three T$_{reg}$ subsets have been identified, which have varying functions, defined by expression of CD45RA, CD25 levels, and Foxp3 [78]. Activated T$_{reg}$s are CD45RA$^-$CD25^{hi}Foxp3^{hi} and show suppressor function; CD45RA$^+$CD25^{moderate}Foxp3^{lo} subset represents resting regulatory T cells without suppressor function; and CD45RA$^-$CD25^{moderate}Foxp3^{lo} cells are non-T$_{reg}$ effector T cells that are capable of cytokine production, making IL-2, IL-17, or IFNγ [78]. In autoimmune conditions, including systemic lupus erythematosus and sarcoidosis, there is an increased ratio CD45RA$^-$CD25$^+$Foxp3^{hi} cells compared to CD45RA$^-$CD25$^+$Foxp3^{lo} cells, although the absolute number of T$_{reg}$ cells is overall reduced. This ratio was found to be reversed in cancer [79].

Th$_{17}$ Compared to Th$_1$ or Th$_2$. Th$_{17}$ cells have bivalent expression of T-bet and GATA-3, allowing them to reprogram into either Th$_1$ or Th$_2$ cells [32, 80]. Th$_{17}$ cells generated by TGFβ and IL-6 *in vitro* can convert into IL-12-producing

Th$_1$ or IL-4-producing Th$_2$ cells when ongoing stimulation with proinflammatory cytokines is not sustained [1]. Th$_1$ cells differentiate from Th$_{17}$ cells *in vitro* when IL-12 is present in medium in the absence of IL-6. IL-17$^+$IFNγ^+-producing cells may represent an intermediate state during Th$_1$ development from Th$_{17}$ precursors. T-bet and RORγT are coexpressed in this transition state, allowing maturation of either the Th$_1$ or Th$_{17}$ lineage [32].

A distinct population of Th$_1$/Th$_{17}$ cells has been identified, which are CD4$^+$ T cells capable of producing IFNγ, GM-CSF, and IL-17 [81]. Th$_1$/Th$_{17}$ cells express both T-bet and RORC2 concurrently to allow for bivalent cytokine production. In an inflammatory environment, they can further become polarized to become Th$_1$/exTh$_{17}$ cells through loss of active transcription from the RORC2 locus. Th$_1$/ex-Th$_{17}$ cells produce IFNγ and GM-CSF but lost the ability to produce IL-17 [81]. Although T$_{reg}$s are capable of transforming into IFNγ and IL-17 producing cells, Th$_1$/Th$_{17}$ cells are a separate entity, as expression of the transcription factor Helios (present in thymic-derived T$_{reg}$s and to a lesser extent in peripherally induced T$_{reg}$s) is absent or low in this subset.

Th$_1$/Th$_{17}$ cells have been associated with promoting autoimmune target organ damage in β-cells of human and animal models of type I diabetes mellitus [82] within synovial tissue of children with juvenile arthritis [83] and within the gastrointestinal tract of patients with Crohn's disease [84]. Blockade of IFNγ with monoclonal antibodies reduced pancreatic β-cell destruction in an animal model, supporting that Th$_{17}$ cells that express a Th$_1$-like phenotype are pathogenic [81].

T$_{FH}$ Compared to T$_{reg}$, Th$_{17}$, or Th$_1$. T$_{FH}$ cells can be differentiated from T$_{reg}$s, which requires loss of Foxp3 expression by T-B cell interaction with costimulation provided by CD40-CD40L interaction [24]. T$_{reg}$s differentiate into T$_{FH}$ cells in Peyer's patches to promote IgA production and mucosal immunity [1]. T$_{FH}$ cells mature from TGFβ-induced T$_{reg}$ precursors in response to IL-21 and chemotaxis through CXCR5 signaling, which homes CD4$^+$ T cells to follicles in secondary lymphoid tissue [23].

Th$_{17}$ and T$_{FH}$ cells both require STAT3 activation and costimulation mediated by ICOS, which increases activation of the transcription factor, c-MAF, involved in both Th$_{17}$ and T$_{FH}$ maturation [85]. In ICOS knockout mice, decreased c-MAF activation prevents development of T$_{FH}$ cells and prevents defects in Th$_{17}$ cells, which produce less IL-17 in response to stimulation [85]. IL-12 is required during early dendritic cell-mediated priming of both Th$_1$ and T$_{FH}$ cells, suggesting that T$_{FH}$ and Th$_1$ cells are derived under common innate immune signals [86].

Th$_2$ Compared to T$_{FH}$ or Th$_9$. The same priming cytokine, IL-4, is required for the development of Th$_2$, T$_{FH}$, and Th$_9$ subsets. Th$_2$ cells can become T$_{FH}$ cells through upregulation of CXCR5 expression for homing to germinal center follicles will continue to produce IL-4 but lose the ability to produce other Th$_2$ cytokines, including IL-5 and IL-13 [13, 48]. T$_{FH}$ cells do not have to progress through a Th$_2$ stage in their development, as they still develop in GATA3 knockout mice [23].

Th$_9$ cells can be derived from Th$_2$ cells when TGFβ is added to cells polarized with IL-4 in culture medium [13]. Continued activation of the Th$_2$ cytokine, IL-4, or the Th$_9$ cytokine, IL-9, in transgenic mice leads to the same phenotype, with asthma and bronchial hyperresponsiveness due to mucosal inflammation, also suggesting interconnectedness in their maturation [87].

A schematic diagram of interactions involved in CD4$^+$ T cell lineage specification is provided in Figure 1.

Naïve CD4$^+$ T cells undergo subset specification based on predominant cytokines and chemokines present within the environment. In the presence of IFNγ and IL-12, naïve CD4$^+$ T cells upregulate STAT1 which induces specification to the Th$_1$ lineage through T-bet expression [9, 58]. Th$_1$ cells then produce IFNγ through STAT4 expression [88]. A positive feedback loop exists to further promote Th$_1$ specification [89]. Conversely, Th$_1$ cells inhibit Th$_2$ specification through IFNγ production, as well as through T-bet expression. Th$_2$ cells develop when IL-4 is present in the environment and require STAT6 upregulation to induce GATA-3 expression within the nucleus [90]. Th$_2$-specified cells produce IL-4, IL-5, and IL-13. When TGFβ is also present in the cytokine microenvironment, Th$_9$ specification can occur [91]. As Th$_1$ cells inhibit Th$_2$ specification, Th$_2$ cells also provide negative feedback to Th$_1$ differentiation through suppressing IL-12 expression [89]. Th$_{25}$ cells, induced by IL-25, represent a separate mechanism of GATA-3 induction and production of Th$_2$-cytokines, IL-4, IL-5, and IL-13, which has been linked to reactive airway inflammation [46]. Th$_{25}$ specification requires expression of the E3 ubiquitin ligase Act1 and the cognate receptor IL-17RB [46], related to the Th$_{17}$ subset. The Th$_{17}$ and T$_{reg}$ lineages are interrelated, and both can be induced in the presence of TGFβ, with Th$_{17}$ specification preferred when IL-6 is also present. Th$_{17}$ cells express lineage-specific transcription factor RORγT as well as other retinoic orphan receptors, which leads to production of Th$_{17}$ cytokines, IL-12, IL-17, and IL-17F [92]. IL-12 produced by Th$_{17}$ cells can provide feedback to promote Th$_1$ specification [1]. In the presence of IL-6 or IL-21, T$_{reg}$ cells can become Th$_{17}$ cells [16]. IL-6 and TNFα can induce STAT3 activation and induce Th$_{22}$ specification, a process that is dependent on the aryl hydrocarbon receptor [19]. In the presence of IL-21, CXCR5, and costimulation by CD40-CD40 ligand interaction, T$_{reg}$s can undergo T follicular helper (T$_{FH}$) specification [23].

3.1. Epigenetic Modifications of Cytokine Loci Determine Lineage Specification.

Flexibility of CD4$^+$ T cells to switch effector cytokine function in response to environmental signals depends on permissiveness of chromatin to transcription factor binding at loci encoding cytokines.

CD4$^+$ T cells undergo multiple cell divisions before producing subset-specific effector cytokines, since maturation of CD4$^+$ T cells into subsets requires continued stimulation under cytokine polarizing conditions, resulting in stable patterns of gene expression due to chromatin remodeling of loci encoding cytokine genes [1].
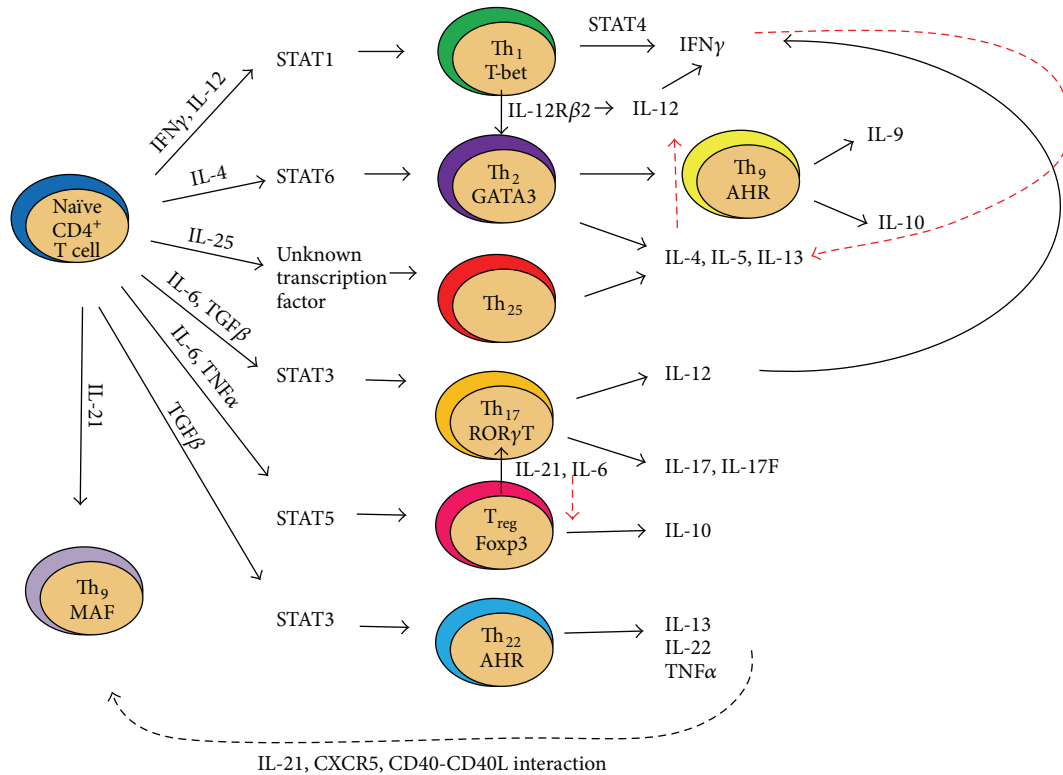
FIGURE 1: Cross talk between CD4$^+$ T cell subsets mediated by effector cytokines.

Epigenetic influences bring alterations of cytokine production profiles through mechanisms including alterations of chromatin structure through histone acetylation or DNA demethylation and modifications of microRNA activity [1]. Histone acetylation or demethylation of DNA brings decondensation ("opening") of chromatin (or conversion of heterochromatin to euchromatin), resulting in increased access for transcription factor binding [4]. Modifications that occur when signals within the extracellular environment change allow a CD4$^+$ T cell to redirect its differentiation program to provide flexibility to shift cytokine production for the optimal clearance of offending pathogens [1].

Genome-wide chromatin immunoprecipitation studies (CHIP) have characterized histone modifications which accompany changes in gene expression within CD4$^+$ T cells. Trimethylation of lysine 4 at histone 3 (H3K4me3) occurs at promoter and enhancer elements of actively expressed genes. Trimethylation of lysine 27 of histone 3 (H3K27me3) represses a locus, and this repression is reversed by histone demethylases [3]. Presence of both of these histone modifications (bivalency of histone 3, or coexpression of H3K4me3 and H3K27me3) allows a gene promoter to become activated or silenced, depending on the signal received [1]. In addition, chromatin looping can bring regulatory elements to proximity with promoters of target genes enabling regulation of gene expression, a process mediated by CCCTC-binding factor, an insulator protein that binds proximal elements of a gene to prevent it from interacting with surrounding chromatin [32]. Looping allows transcriptional regulatory elements to

reposition in the nucleus during T cell maturation to promote or repress transcription [7]. Chromatin looping allows the Th$_2$ cytokine locus control region (LCR) to form a complex with the promoters that induce IL-4, IL-5, and IL-13 cytokine expression for activation of the Th$_2$ transcriptional program [64].

In addition to changes in chromatin structure, interchromosomal associations allow for regulation of effector cytokine expression. Chromosome conformation capture studies (3C technique) have identified an interchromosomal interaction between the IFNγ promoter of human chromosome 10 and the LCR of the Th$_2$ cytokine locus on chromosome 11 [64]. A chromatin hub configuration between Th$_1$ and Th$_2$ cytokine loci primes a naïve CD4$^+$ T cell to produce either Th$_1$ or Th$_2$ cytokines 1 hour after TCR activation. After this early wave of cytokine production activation of STAT proteins is required for maintenance of the signal [62].

The lineage-specific transcription factors that direct Th$_1$, Th$_2$, and Th$_{17}$ commitment (Tbx21, GATA3, and RORγT, resp.) carry bivalent epigenetic marks, signifying the possibility of subset specification reversibility. However, Foxp3 expression in T$_{reg}$ cells is univalent, suggesting subset differentiation is possible but not reversible [32, 93].

3.2. mTOR as a Regulator of CD4$^+$ Differentiation. The serine-threonine kinase, mammalian target of rapamycin (mTOR), is a candidate gene as a master regulator of CD4$^+$ T cell differentiation and metabolism [94], which are interconnected. mTOR activation in CD4$^+$ T cells has diverse roles in

regulation of cell growth and proliferation, mRNA turnover and transcription, translation, regulation of vesicular traffic, autophagy and amino acid recycling, cytoskeletal reorganization, and control of cell size [95]. It exerts its function through phosphorylation of its target substrates, such as p70 S6 kinase and 4E-BP1 (which regulate translation), and DAP1 (which inhibits induction of autophagy). mTOR is positively regulated by the GTPase Rheb and negatively regulated by the tuberous sclerosis complex (TSC) [96].

mTOR activation results in increased maturation of $CD4^+$ T cells into effector cells, with reduction of Foxp3 expression and T_{reg} generation [97]. Absence of mTOR at the DP phase in T cell development abrogates the ability to produce Th_1, Th_2, or Th_{17} cells and results in a high proportion of thymocytes maturing as T_{reg}s [26]. This maturation defect is associated with decreased activation of STAT 4, 6, and 3, resulting in failure to upregulate lineage-specific transcription factors T-bet, GATA3, and RORγT [26]. Consistently, treatment with rapamycin (an mTOR inhibitor) results in thymic involution, decreased egress of T cells, and blockade of the DN to DP transition of T cell development [94]. Conversely, in effector $CD4^+$ T cells, activation of the sphingosine-1-phosphate ($S1P_1$) signaling pathway results in increased mTOR-Akt activation, which inhibits intrathymic generation and suppressor function of T_{reg}s [98, 99]. Therefore, mTOR overexpression renders effector $CD4^+$ T cells resistant to T_{reg} suppressor activity [94].

During effector $CD4^+$ T cell activation, the PI3K-Akt-mTOR pathway is activated and regulates the effector T cell/T_{reg} cell fate decision [99]. mTOR activation transmits signaling through the IL-2 receptor, and IL-2 binding to the IL-2 receptor prevents T cell anergy, required for maintenance of effector T cells [25]. T_{reg} cells do not rely on mTOR activation for IL-2 receptor signaling but instead use an alternate PIM2-dependent pathway for IL-2 signaling, as STAT5 increases IL-2 through activation of PIM2. The activity of mTORC1 in comparison to mTORC2 determines whether naïve T cells differentiate into the Th_1/Th_{17} lineage versus Th_2 cells [100]. In the absence of Rheb, a kinase required for the function of mTORC1, Th_1 and Th_{17} cells are not produced, as mTORC1 is required for Th_1/Th_{17} specification [100]. In the absence of rictor, a component of mTORC2, Th_1, and Th_{17} cells is generated but not Th_2 cells. Therefore, mTORC1 activity is required to generate Th_1 cells, while mTORC2 activity is required for Th_2 specification [100].

Lineage determination by mTOR is through induction of changes in cellular metabolism through activation of its substrates (hypoxia-inducible factor, sterol regulatory element binding proteins 1 and 2), through regulation of mitochondrial function, and through negative regulation of autophagy [101]. mTOR activation results in a stimulation of glycolysis, pentose phosphate shunt pathway activity (oxidative branch), and lipid biosynthesis with a concurrent reduction in fatty acid oxidation [101]. Since mTOR stimulates glycolytic metabolism, it promotes Th_1, Th_2, and Th_{17} specification, as these subsets have high metabolic requirements [102]. As such, Th_1, Th_2, and Th_{17} cells also show high expression of the Glut1 receptor to facilitate increased glucose transport

[102]. As mTORC1 supports an anabolic state with lipid biosynthesis over its utilization/oxidation, this results in reduced AMPK activity [103]. AMPK activity is critical to T_{reg} metabolism, supporting effector over T_{reg} cell specification when expression is low [103]. mTOR additionally impacts aerobic metabolism through control over mitochondrial function through regulation of mitochondrial number [104], transmembrane potential [105], oxygen consumption [105, 106], and autophagy [107–109].

A model of $CD4^+$ T cell specification has been proposed based on differential activation of mTOR. In this model, the generation of effector $CD4^+$ T cells from naïve $CD4^+$ T cell precursors is dependent on mTOR-mediated induction of metabolic programs within $CD4^+$ T cells [110]. mTOR^{hi} and mTOR^{lo} naïve $CD4^+$ T cells were found to have different fates [111]. mTOR^{hi} $CD4^+$ T cells will become effector cells, while mTOR^{lo} $CD4^+$ T cells represent a long-lived $CD4^+$ T cell population, with expression of Bcl-2, CD62L, and CD25 and a higher propensity to develop into T_{reg}s [111]. Interestingly, the mTOR^{lo} and mTOR^{hi} naïve T cell populations can be separated based on their size, with the mTOR^{hi} population having increased cell size [111].

Although mTOR activity is critical to the regulation of $CD4^+$ T cell development, specification, and metabolism, its hyperactivation is pathogenic. mTOR complex I is overexpressed in autoimmune diseases, genetic cancer syndromes, and obesity which correlates with a reduction in suppressor T_{reg}s [112]. mTOR stimulates aerobic glycolysis, which promotes the Warburg effect within tumors [112]. mTORC1 activity increases Th_{17} cell number and reduces T_{reg}s in systemic lupus erythematosus (SLE) [113, 114]. Overexpression of mTOR results in defects in macroautophagy [105, 115], which is pathogenic through mitochondrial dysfunction, ATP depletion, and increased oxidative stress [116]. mTOR inhibitors, such as rapamycin, are therefore therapeutic through inhibiting aberrant mTOR activity in the treatment of autoimmunity and malignancies [117–119].

4. Conclusion

As mentioned, naïve $CD4^+$ T cells mature into Th_1, Th_2, Th_9, T_{FH}, Th_{17}, or T_{reg} subsets in response to innate immune signals, costimulatory interactions with APCs, paracrine cytokine signals, and through mTOR-mediated changes in energy metabolism [31, 120]. The resulting $CD4^+$ T cell subsets are highly plastic with numerous transitory populations identified that are capable of heterogeneous cytokine production as well as the ability to cross talk with other naïve, effector, memory, and regulatory $CD4^+$ T cells. With continued stimulation, $CD4^+$ T cells develop patterns of stable cytokine expression, yet chromatin remodeling alters cytokine programs in subsets containing bivalent chromatin modifications at loci encoding lineage-specific transcription factors to maintain the capability of shifting their phenotype in response to environmental alterations [32]. mTOR was recently identified as a possible master regulator of $CD4^+$ T cell differentiation [26] and exerts $CD4^+$ T cell specification through alterations in cellular metabolism. An improved

understanding of how to modulate CD4$^+$ T cell pools through inducing phenotypic shifts could provide wide health benefits from limiting autoimmune responses to optimizing antitumor immune responses and represents an exciting area of investigation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding publication of this paper.

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