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# Do Memory CD4 T Cells Keep Their Cell-Type Programming: Plasticity versus Fate Commitment?

## Epigenome: A Dynamic Vehicle for Transmitting and Recording Cytokine Signaling

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$CD4^+$ T cells are critical for the elimination of an immense array of microbial pathogens. Although there are aspects of helper T-cell differentiation that can be modeled as a classic cell-fate commitment, $CD4^+$ T cells also maintain considerable flexibility in their transcriptional program. Here, we present an overview of chromatin biology during cellular reprogramming and, within this context, envision how the scope of cellular reprogramming may be expanded to further our understanding of the controversy surrounding $CD4^+$ T lymphocyte plasticity or determinism.

OUTLINE



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The genetic information encoded in DNA is presented in the context of chromatin, a complex of nucleic acid and associated proteins called histones. The dynamic nature of the chromatin is a defining feature of the epigenetic regulation. The regulation of $CD4^+$ T cells similar to any other cell type is therefore tightly linked with the epigenome—the combinatorial variance in localization and modifications of chromatin factors and underlying DNA. Propelled by rapid technological advances in sequencing, the field of epigenomics is enjoying unprecedented growth with no sign of deceleration (Rivera and Ren 2013). An expanding number of immunologists are working to explore exciting frontiers in epigenomic regulation of immune cells. Consequently, the number of data sets and publications has grown in recent years. The theme emerging from these genome-scale studies is that the chromatin structure is an attractive template for both *transmitting* and *recording* immunological events. For example, the demethylation of the PD-1 promoter is recorded during the effector phase of T-cell exhaustion (Youngblood et al. 2011), whereas cytokine signaling in $CD4^+$ T helper cells can be transmitted through gain and loss of histone acetylation at thousands of genomic regions (Vahedi et al. 2012, 2015). Here, we aim to discuss the controversy surrounding $CD4^+$ T lymphocyte plasticity or determinism mostly from a chromatin biology perspective. This article has a three-prong focus: We start by discussing how cell fate is regulated during development. We then focus on reprogramming studies and the characteristics of factors capable of inducing cell-fate changes. And finally we evaluate $CD4^+$ T-cell plasticity from the chromatin biology perspective.

## HOW IS CELL FATE DETERMINED?

Cellular-state information between generations of developing cells is propagated through the epigenome particularly via the accessible parts of the chromatin. Consistent patterns of gain and loss of chromatin accessibility has been reported as cells progress from embryonic stem cells to terminal fates (Stergachis et al. 2013). Chromatin accessibility together with covalent modifications of histones is controlled in large part by the action of multiple transcription factors that recognize and bind specific sequences in the genome. Rather than one regulatory protein being singularly responsible for creating the chromatin state and determining each cell type, particular combinations of transcription factors elicit cell-fate changes and maintain cell identity (Iwafuchi-Doi and Zaret 2016). The emerging theme from recent studies is that the chromatin state has a hierarchical organization where every layer is regulated by distinct groups of transcription factors. The first layer relates to a small number of transcription factors who act first on the chromatin and are referred to as "pioneer transcription factors." These factors are distinguished from other transcription factors by their ability to bind their cognate DNA sites directly on the nucleosome, even in chromatin that is locally compacted by linker histone (Zaret and Mango 2016).

Such pioneer transcription factors initiate cooperative interactions with other transcription factors to elicit changes in local chromatin structure. A second layer in this hierarchical organization consists of transcription factors such as AP-1 family members JUNB, BATF, and IRF4, which further prime parts that later become associated with more specific and dynamic factors (Garber et al. 2012; Kurachi et al. 2014). The bottom layer, particularly essential for $CD4^+$ T-cell biology, relates to transcription factors downstream of signal transduction pathways. These signal-dependent transcription factors are dynamic and control more specific sets of genes that have common biological functions. For instance, the signal transducers and activators of transcription (STAT) proteins target the late induced T-helper-specific chromatin states, while the nuclear factor κB (NF-κB) factors Rel, Relb, and NFKB1 target the inflammatory program (Garber et al. 2012; Vahedi et al. 2012; Ostuni et al. 2013).

## HOW FIXED IS THE DIFFERENTIATED STATE?

The prevailing paradigm in cell development has been that somatic cells become irreversibly committed to their fate and lose potency as they specialize. Despite physiological settings, exper-



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iments performed several decades ago showed that the fusion of different pairs of cell types can awaken dormant gene-expression programs (Blau 1989). Subsequently, breakthroughs in cellular reprogramming techniques, recognized by a Nobel Prize, convinced us that plasticity can be induced and "fate" can be changed. Reprogramming studies on the use of transcription factors to interconvert cells of different types brought to light the distinguished role of pioneer factors. Direct assessments of the initial binding of the induced pluripotency factors Oct4, Sox2, Klf4, and c-Myc showed that Oct4 and Sox2, and to a lesser extent Klf4 target predominantly silent and inaccessible chromatin—resistant to DNaseI—when they are forcibly expressed in human fibroblast cells (Soufi et al. 2012). Studies in immune cells also showed that when C/EBPa is ectopically expressed in pre-B cells, it can convert B cells to macrophages by targeting closed chromatin (van Oevelen et al. 2015). Together, cell fate can be altered experimentally as a result of forced expression of various combinations of transcription factors typically including pioneer factors.

## CELL FATE IN CD4$^+$ T CELLS

We have reviewed concepts in development and reprogramming to highlight that CD4$^+$ T cells, like all other cells, are packaged into chromatin. Specialized CD4$^+$ T cells (e.g., T helper 1 cells), maintain their fate until environmental changes lead to the expression of "influential" transcription factors that can potentiate cells to change their program. Given the stability of the differentiated state in vivo, an understanding of the regulation of CD4$^+$ T-cell differentiation by mechanisms that allow the type of plasticity observed in reprogramming is critical for our understanding of vaccines and immune memory.

Early in vitro studies argued that once a CD4$^+$ T cell had chosen its fate, it would not easily switch to another fate, even if exposed to the cytokines that drove differentiation to the opposing subset (Mosmann and Coffman 1989; O'Shea and Paul 2010; Yamane and Paul 2012). This dogma has been challenged over the last decade because the possibility of polarized T cells to repolarize toward mixed or alternative fates have been reported (DuPage and Bluestone 2016). For example, recent lineage-tracing systems in mice showed that endogenously polarized CD4$^+$ T cells from many subsets can alter their phenotype during their life span (Zhou et al. 2009b; Hirota et al. 2011; Wilhelm et al. 2011; Ahlfors et al. 2014). Furthermore, the phenotypic plasticity of regulatory T cells that mirrors each T helper cell subset supports a hypothesis of an inherent flexibility of T cells, both inflammatory and regulatory, to adapt their function to changing environments (DuPage and Bluestone 2016). These in vitro and in vivo studies, extensively reviewed elsewhere (Wilson et al. 2009; Zhou et al. 2009a; Murphy and Stockinger 2010; O'Shea and Paul 2010; DuPage and Bluestone 2016), suggest that CD4$^+$ T cells are adaptable and can show phenotypic plasticity in response to changing contexts.

## REGULATION OF PLASTICITY IN CD4$^+$ T CELLS

In this perspective, we propose that plasticity of CD4$^+$ T cells works by the same mechanism that initially selected the first fate. If a cell population is able to respond to new signals because it expresses that specific extracellular receptor, has the cytoplasmic means of signaling, and the ability to induce the transcription factors that have strong impact on the chromatin, then the cellular identity at the epigenetic level can be modified to allow the cell to take on new identity and function.

The CD4$^+$ T cell's initial activation through T-cell receptor (TCR) results in the activation of primed elements rather than selection of new regions (Allison et al. 2016). The presence of polarizing cytokines initiates the process of selecting the T-cell fate with genome-scale epigenetic changes (Ciofani et al. 2012; Vahedi et al. 2012). In the face of a new challenge or extracellular environment, the presence of new cytokine signals is propagated through available cytokine receptors and cytoplasmic signaling events into the nucleus. These events induce expression of transcription factors with varying degrees of intrinsic ability to modify the chromatin environment



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(Vahedi et al. 2012). Although branding transcription factors to "master" regulators attracted a lot of attention among T-cell biologists (Rothenberg 2014), we propose to set aside the semantics and what the words "pioneer" or "master" may imply. Identifying T-helper-specific transcription factors with an intrinsic ability to bind silent chromatin and consequently alter T-cell fate has an important implication for understanding T-cell plasticity.

## INTEGRATING GENOMIC DATASETS FOR A BETTER UNDERSTANDING OF SUBSETS

Deciphering mechanisms of $CD4^+$ T-cell plasticity requires a more precise definition of these same subsets (Fig. 1). We argue that this is because populations of cells may appear to fit into a certain subset based on traditional measures (i.e., flow cytometry), but is actually composed of cells with different histories of cytokine exposure that has been imprinted onto the chromatin. These differences may not be immediately apparent, but may have major influence on cellular function in the right context. Highly parametric multidimensional measurements of T cells by mass or flow cytometry has revealed an overwhelming number of subpopulations in any given subset during an immune response (Newell et al. 2012; Gaudilliere et al. 2014; O'Gorman et al. 2015). The challenge remains in determining whether these states are transi-

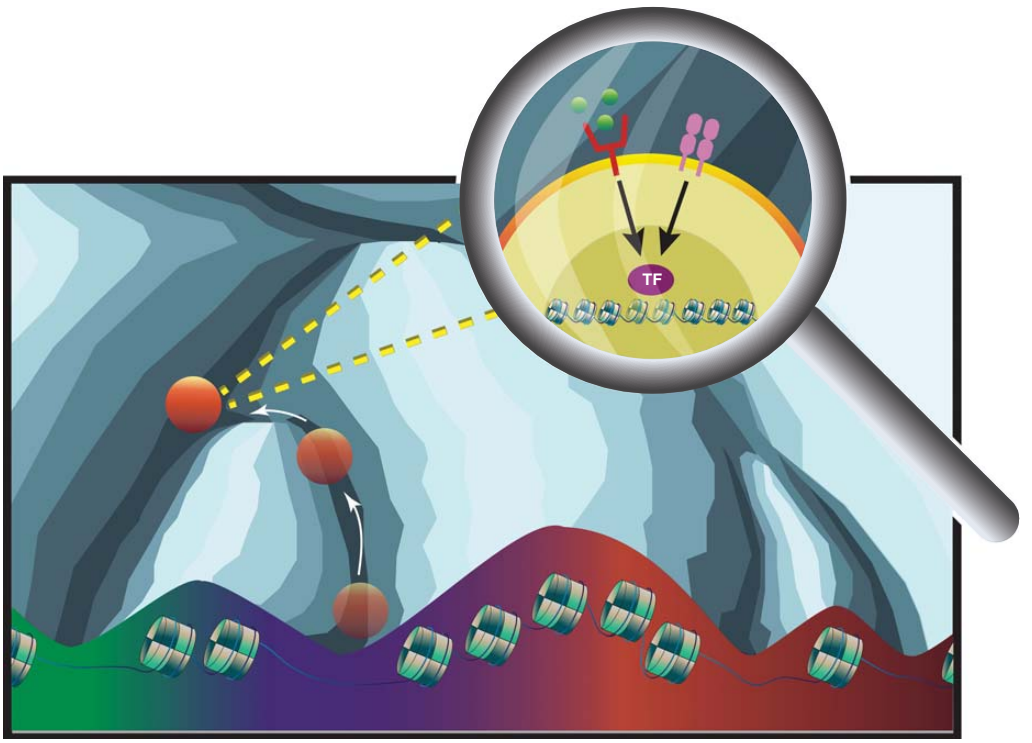


**Figure 1.** $CD4^+$ T-cell plasticity regulated by transcription factors (TF) that control the accessible chromatin. The process of differentiation lowers the potential of the cell to differentiate to other lineages as visualized by Waddington's landscape. The cell eventually becomes committed to a lineage and resides in the valleys of accessible chromatin. New signaling events, through T-cell receptor (TCR) signaling and extracellular receptors, are propagated to the nucleus through the appropriate cytoplasmic machinery. This causes the expression of transcription factors that modulate chromatin accessibility to allow for the cell to obtain new cell identity and function. Epigenetic regulatory mechanisms such as DNA methylation and histone modifications can either enhance or inhibit this process.



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tory or stable and to determine their functional status or disease relevance.

Integrating epigenetic measurements is an attractive solution for more precise definitions of cell identity. The relative stability of cell identity conferred by epigenetic mechanisms at the genome-wide level makes this possible. Chromatin accessibility measurements by DNase I hypersensitivity or the new and robust assay ATAC-seq has provided an excellent foundation of cell identity. A remarkable example is a recent comparison of chromatin accessibility landscapes in innate and adaptive T cells revealing similarity and differences in these two distinct cell types (Shih et al. 2016).

## SINGLE-CELL HETEROGENEITY

Although not a novel observation, cellular heterogeneity in once seemingly homogenous populations is even more apparent with the advent of new technologies to take single-cell measurements. Variability in canonical subsets may help fill gaps in proposed links between certain CD4 subsets and disease. Heterogeneity may not be ultimately meaningful in most tissue systems in multicellular organisms, as a certain level of homeostasis is required. However, the immune system is one environment in which cellular heterogeneity is of higher priority. Rare cells with the ability to respond to ever-changing pathogens provide the edge necessary to overcome these pathogens. Indeed, heterogeneity of the lymphocyte compartment encoded in the diverse antigen receptor repertoire has granted us a mechanism that ensures a small proportion of the lymphocyte population will have the means of recognition for nearly any new pathogen. Much attention is being focused on deconstructing the signaling and epigenetic mechanisms that give rise to variability and to assess the impact of that variability in the host immune response. As a result of recent genomics tools such as single-cell RNA-seq (Kolodziejczyk et al. 2015) and ATAC-seq (Buenrostro et al. 2015) together with revolutionary single-cell proteomics, we are poised to improve our understanding of cell identity from a single-cell perspective.

## LINEAGE TRACING IS IMPORTANT IN DETERMINING PAST, PRESENT, AND FUTURE

We posit that snapshot measurements of the CD4 compartment in response to an immune stimulus does not allow us to adequately resolve multiple underlying populations at the epigenetic level. As discussed above, the influence of previous cytokine exposure can be recorded in the chromatin. Understanding how this history may influence future behavior and how a seemingly homogenous population of cells might diverge in response to new stimuli is critically important. If the goal is to understand the transcriptional circuitry that leads to polarized subsets of CD4 T cells, it stands to reason that understanding how these epigenetics states corresponds to function is of equal importance. Addressing these questions will not only require an integration of genomics and phenotypic data, but also better methods of lineage tracing. The advent of CRISPR-Cas9 brings the ability to genetically track cells in an ongoing manner and to serve as signposts from which we can take our snapshot measurements of the cell to understand its trajectory (McKenna et al. 2016).

## CONCLUDING REMARKS

Initiated by the application of microarrays, the last decade unraveled the battery of transcription factors essential in T-helper-cell differentiation. Yet, we have limited knowledge about the efficacy of these proteins on the chromatin and their relative ability to alter the accessibility landscapes and ultimately T-cell fate in the periphery. Novel techniques in genomics, epigenomics, proteomics, single-cell biology, and gene editing together with computational techniques developed for data integration can help us fill these gaps. Understanding the mechanisms underlying plasticity in $CD4^+$ T cells will provide us with the opportunity to tailor the $CD4^+$ responses for better vaccination and to reinvigorate T-cell responses in chronic infection and cancer. Overcoming the stable and defined chromatin state through the expression of transcription factors with effectuating prop-



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erties can potentially overcome mechanisms of inhibition and allows new signals—and fates—to be propagated to the nucleus to ultimately control the outcome of the immune response.

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# Do Memory CD4 T Cells Keep Their Cell-Type Programming: Plasticity versus Fate Commitment?

## T-Cell Heterogeneity, Plasticity, and Selection in Humans

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The wide range of effector and memory T cells is instrumental for immune regulation and tailored mechanisms of protection against pathogens. Here, we will focus on human CD4 T cells and discuss T-cell plasticity and intraclonal diversification in the context of a progressive and selective model of CD4 T-cell differentiation.

OUTLINE



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CD4 T cells are born naïve and upon antigenic stimulation in secondary lymphoid organs differentiate to effector T cells and acquire the capacity to migrate to peripheral tissue and to produce a range of cytokines that mediate effector responses directly and through the activation of innate immune cells. $T_H1$ cells produce interferon γ (IFN-γ) that activates macrophages to kill intracellular bacteria, whereas $T_H2$ cells produce interleukin (IL)-4 and IL-5, which activate mast cells, basophils, and eosinophils in response to helminthes, and $T_H17$ cells produce IL-17 and IL-22, which drive recruitment of neutrophils and production of antimicrobial peptides in response to extracellular bacteria and fungi. $T_H$ cells that migrate to B-cell follicles differentiate to follicular helper T cells ($T_{FH}$) and provide help to B cells for antibody production. Following antigen-driven expansion, some T cells survive as circulating central memory T cells ($T_{CM}$) and effector memory T cells ($T_{EM}$) to provide immune surveillance in lymph nodes and peripheral tissues; others that seed nonlymphoid tissues give rise to a population of tissue-resident memory T cells ($T_{RM}$) to provide immediate protection at sites of pathogen reentry.

The heterogeneity of effector and memory CD4 T cells raises questions about the nature of differentiation signals, the extent of fate diversity, the lineage relationship between different fates, and the degree of plasticity at different stages of differentiation. Here we will summarize studies performed in the human system that have advanced our understanding of CD4 T-cell heterogeneity and discuss T-cell plasticity and intraclonal diversification in the context of a progressive and selective model of CD4 T-cell differentiation.

## HETEROGENEITY OF HUMAN CD4 T CELLS

The extent and nature of naïve T-cell differentiation is determined by signals provided by the pathogen and by the local environment where priming occurs (Medzhitov 2007). By promoting expression of master transcription factors, cytokines produced by dendritic cells and other innate immune cells represent key determinants of $T_H$ cell differentiation. Classical examples are IFN-γ and IL-12, which induce through STAT1 and STAT4 expression of T-bet that drives the $T_H1$ program. Similarly, IL-4 through STAT6 induces expression of GATA-3 that drives the $T_H2$ program, while IL-6 through STAT3 induces RORγt that drives the $T_H17$ program. Importantly, cytokines act in combination with signals from the T-cell antigen receptor (TCR) and costimulatory molecules as well as signals triggered by tissue-specific cues and environmental factors—such as nutrients, vitamins, and even pollutants and salt—to define internal transcriptional landscapes that confer T-cell identity (Bonelli et al. 2014). Some signals trigger internal circuits that enforce, reinforce, or destabilize T-cell polarization through multiple mechanisms, including epigenetic modifications (Wang et al. 2015). MicroRNAs and long noncoding RNAs (lncRNAs) have also been found to control various aspects of CD4 T-cell differentiation and effector function (Baumjohann and Ansel 2013; Panzeri et al. 2015).

Mouse models have been used in a reductionist approach to define the contribution of single elements and signaling pathways involved in CD4 T-cell differentiation and to evaluate the role of different types of polarized $T_H$ cells in protection or immunopathology. Human studies are providing important clues as to the extent of T-cell fate diversity induced by different pathogens in different tissues (Farber et al. 2014; Sallusto 2016). An example is represented by studies analyzing the memory T-cell response induced by extracellular pathogens that elicit two distinct types of $T_H17$ cells. T cells primed by *Staphylococcus aureus* produce IL-17 and the immune regulatory cytokine IL-10. In contrast, T cells primed by *Candida albicans* have a more inflammatory phenotype, producing IL-17 together with IFN-γ. This difference was found to be dependent on the cytokines elicited by the different pathogens, primarily IL-1β (Zielinski et al. 2012). Another example is provided by CD4 T cells in the skin of patients with atopic dermatitis or psoriasis that revealed the existence of T cells producing IL-22 but not IL-17 (Eyerich et al. 2009). These $T_H22$ cells can be also found in the blood, where they express



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CCR6 and the skin-homing receptors CLA and CCR10, and can differentiate from naïve T cells in a process that requires the transcription factor AHR and signals from vitamin D3 (Duhen et al. 2009; Trifari et al. 2009). Yet other examples include IL-9-producing $T_H9$ cells, initially defined in mice (Veldhoen et al. 2008) that are also increased in the skin lesions of psoriasis (Schlapbach et al. 2014) and granulocyte macrophage colony-stimulating factor (GM-CSF)-only-producing $T_H$ cells (Noster et al. 2014).

Studies on the human T-cell response induced by *Mycobacterium tuberculosis* led to the discovery of a distinct type of IFN-γ-producing $T_H1$ cells (that we defined as $T_H1^*$) that can be distinguished from virus-induced $T_H1$ cells based on the expression of chemokine receptors and transcription factors (Acosta-Rodriguez et al. 2007; Lindestam Arlehamn et al. 2013). $T_H1^*$ cells express CXCR3 and T-bet, as classic $T_H1$ cells, but also CCR6 and RORγt, which are characteristic of $T_H17$ cells (Fig. 1). Interestingly, $T_H1^*$ development, but not $T_H1$ development, is impaired in patients with *RORC* loss-of-function mutations (Okada et al. 2015), a finding that supports the existence of distinct pathways of $T_H1$ cell differentiation. The signals present in the context of bacterial infections that induce $T_H1^*$ cells remain to be defined. These cells may derive directly from naïve T cells in a RORγt-dependent fashion or from $T_H17$ cells that convert to $T_H1^*$ under the influence of IL-12, TNF-α, and/or IL-1β. The latter possibility is consistent with the finding that in $T_H1^*$ cells, like in $T_H17$ cells, *RORC2* and *IL17A* are demethylated (Mazzoni et al. 2015).

A regulated cytokine production is required for the proper elimination of microbial pathogens, for instance IFN-γ production by $T_H1$ cells for intracellular microbes and IL-17 by $T_H17$ cells for *C. albicans*. However, when analyzed in more detail, microbe-specific memory T cells are found not only in the expected subset but also, at lower frequencies, in other subsets that have different, and even divergent, functions (Becattini et al. 2015). For instance, *C. albicans*–specific T cells are mainly $T_H17$, but some are $T_H1$, $T_H1^*$, or $T_H2$. Similarly,

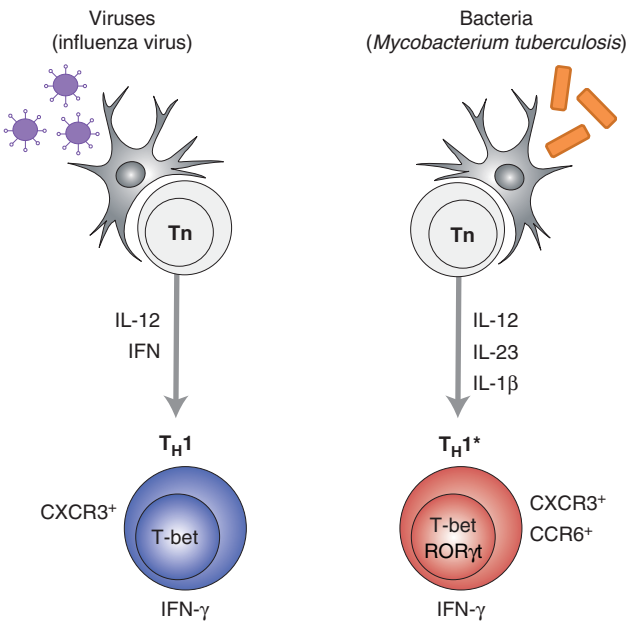


**Figure 1.** Two types of human $T_H1$ cells. $CXCR3^+CCR6^-$ $T_H1$ cells expressing T-bet are preferentially elicited by viruses, whereas $CXCR3^+CCR6^+$ $T_H1^*$ cells expressing T-bet and RORγt are preferentially elicited by bacteria, possibly by different polarizing cytokines present at sites of induction. IFN, Interferon; IL, interleukin.



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*Mycobacteria*-specific T cells are primarily $T_H1^*$, but a few are $T_H1$ or $T_H17$. The heterogeneity of T-cell fates revealed by human studies is not surprising considering the continuous environmental exposure to commensal and pathogenic microbes and the accumulation of memory T cells that have undergone repeated encounters with antigen over several years. Although it is possible that these heterogeneous fates are the result of differential priming, it is also possible that they represent distinct stages of differentiation, owing to the property of T cells to acquire additional functions or to be reprogrammed to alternative fates if exposed to appropriate stimuli, a property that has been defined as T-cell plasticity.

## PLASTICITY AND FATE COMMITMENT: HUMAN STUDIES

Soon after the seminal discovery of $T_H1$ and $T_H2$ cells (Mosmann et al. 1986), some studies both in mice and humans reported the existence of CD4 T-cell clones with a mixed cytokine secretion pattern (Maggi et al. 1988; Umetsu et al. 1988; Street et al. 1990; Openshaw et al. 1995). These $T_H0$ clones producing IFN-γ and IL-4 cells were suggested to be the precursors of $T_H1$ and $T_H2$ cells (Street et al. 1990). This phenomenon was later analyzed by studying epigenetic modifications at cytokine gene loci in naturally occurring human memory $T_H1$ and $T_H2$ cells (Messi et al. 2003). It was found that memory $T_H1$ cells display acetylated histones at the *IFNG* promoter, but not at the *IL4* promoter, whereas reciprocally memory $T_H2$ cells display acetylated histones at the *IL4* but not *IFNG* promoter. However, the hypoacetylation of the nonexpressed cytokine gene did not lead to its irreversible silencing because, on stimulation under $T_H2$ conditions, $T_H1$ cells up-regulated GATA-3 and acquired *IL4* acetylation and expression while continuing to produce IFN-γ, thus becoming $T_H0$ cells. Reciprocally, when stimulated in the presence of IL-12, most $T_H2$ cells up-regulated T-bet and acquired *IFNG* acetylation and expression while continuing to produce IL-4. These findings indicate that most in vivo–primed human $T_H1$ and $T_H2$ cells maintain both memory and flexibility of cytokine gene expression.

Several studies have now provided convincing evidence that most $T_H$ cells, in particular $T_H17$ cells, have a great degree of flexibility. For instance, $T_H17$ cells from the synovial fluid of oligoarticular-onset juvenile idiopathic arthritic patients can shift in vitro from the $T_H17$ to the $T_H17/T_H1$ or $T_H1^*$ phenotype (Cosmi et al. 2011). In patients with allergic asthma, $T_H17$ cells also produce IL-4, suggesting a shift from $T_H17$ to $T_H17/T_H2$ mixed phenotype. Finally, IL-1β can induce conversion of IL-$10^+$ $T_H17$ into more inflammatory IFN-$\gamma^+$ $T_H17$ cells (Zielinski et al. 2012). These examples of flexibility in cytokine gene expression underline the robust and adaptive behavior of effector T cells in the immune response.

There is growing evidence that plasticity is maximal at early stages of differentiation. Consistent with this notion, circulating $CCR7^+$ $T_{CM}$ cells and $CXCR5^+$ $T_{FH}$-like cells, which represent subsets of less differentiated T cells characterized by hypoacetylated cytokine genes, have the potential to differentiate to either $T_H1$ or $T_H2$ when appropriately stimulated (Messi et al. 2003; Rivino et al. 2004). Plasticity in $T_{FH}$ cells is consistent with the finding of subsets of $CXCR5^+$ T cells that have characteristics of $T_H1$, $T_H2$, or $T_H17$ cells (Morita et al. 2011). Plasticity can be progressively lost as cells reach terminal differentiation stages. For instance, $T_{EM}$ cells expressing $CRT_H2$ and producing high levels of IL-4 are unable to up-regulate T-bet and to acquire IFN-γ-producing capacity (Messi et al. 2003). Although irreversible commitment appears to be the exception rather than the rule, these findings suggest that certain conditions of stimulations in vivo exist that lead to irreversible commitment. These conditions may be found at special sites in the body or may develop in particular pathologic situations. Sites of chronic inflammation may represent antigen and cytokine rich environments in which effector T cells are continuously stimulated. Indeed, repeated stimulations of mouse CD4 T cells in the presence of IL-12 or IL-4 enhance cytokine production and lead to strongly polarized $T_H1$ or $T_H2$ cells (Murphy



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et al. 1996). It would be interesting to determine whether reduced plasticity is a characteristic of tissue-resident memory T cells, which in humans also represent a prominent subset of differentiated T cells (Thome and Farber 2015). Further studies are required to assess the plasticity of T cells at different stages of differentiation and in different anatomical compartments.

It is important to consider that plasticity is influenced by the expression of cytokine receptors and transcription factors in the responding T cells and by the availability of polarizing signals in the tissue where antigen reencounter occurs. For instance, $T_H17$ cells that express IL-12R and IL-1R can acquire a $T_H1^*$ phenotype in response to microbes that elicit production of IL-12 or IL-1, a situation that may be found in the course of coinfections. It can be speculated that, in the context of challenge by a related pathogen in a different tissue, the property of plasticity may confer an evolutionary advantage.

## T-CELL PLASTICITY AND INTRACLONAL DIVERSIFICATION

The finding that the human T-cell response, even to a single microbial pathogen, is heterogeneous could be explained by priming of multiple clonotypes, each undergoing a distinct differentiation pathway determined by the nature and strength of antigen, costimulatory, and cytokine signals. Alternatively, it is possible that within an expanding T-cell clone the proliferating cells might acquire different fates as the consequence of stochastic stimulation and plasticity. The "one cell–one fate" and "one cell–multiple fates" models were tested in humans by analyzing the distribution of clonotypes within memory T-cell subsets (Becattini et al. 2015).

By combining antigenic stimulation and TCR deep sequencing on isolated subsets of $T_H1$, $T_H2$, $T_H1^*$, and $T_H17$ memory T cells, it was shown that the same clonotype could be found in different subsets. *C. albicans*–specific clonotypes present in the $T_H17$ subset were also found in the $T_H1^*$ and $T_H2$ subsets (Fig. 2A). This finding was strengthened by the isolation of T-cell clones with identical TCR αβ, but different surface marker and effector function, and by in vitro priming experiments showing that a single naïve T cell could generate multiple fates in one round of stimulation with *C. albicans* (Becattini et al. 2015). The intraclonal differentiation toward multiple fates was even more striking in the case of memory T cells induced by the tetanus toxoid vaccine with several TCR Vβ sequences being found in $T_H1$, $T_H2$, $T_H1^*$, and $T_H17$ subsets (Fig. 2B).

Given the overwhelming evidence for intraclonal diversification, how can we explain highly polarized responses to pathogens? Interestingly, the dominant $T_H17$ response induced in vivo by *C. albicans* was because the clones within this subset were more expanded, rather than more numerous, as compared to the clones in the other subsets. This finding would be consistent with the initial priming of multiple types of T cells, followed by the selective expansion of $T_H17$ cells, which may be determined by the prevailing cytokines at the site of priming or in the tissue where the pathogen is encountered. Interestingly, this was not the case for T cells primed by a weak vaccine such as tetanus toxoid that failed to select for a particular type of polarized cells. The generation of a range of effector and memory T cells within the same clone may be consistent with an early diversification or with the priming of uncommitted CD4 T-cell clones that subsequently progress along multiple differentiation pathways (Lanzavecchia and Sallusto 2000).

In the context of heterogeneous T-cell responses, an important question that needs to be addressed is whether the generation of functionally diverse T-cell subsets is advantageous for the host. Although failure to generate the right class of $T_H$ cell response may lead to susceptibility to infections, it is possible that the induction of a spectrum of effector and memory T cells endowed with different migratory capacities would provide the host with a range of differentiated precursors to be recruited and expanded where necessary.

Moving ahead, the challenge is to dissect in terms of time and space the events that determine the functional heterogeneity of T-cell responses in humans. Improved technologies for obtaining information on antigen specific-



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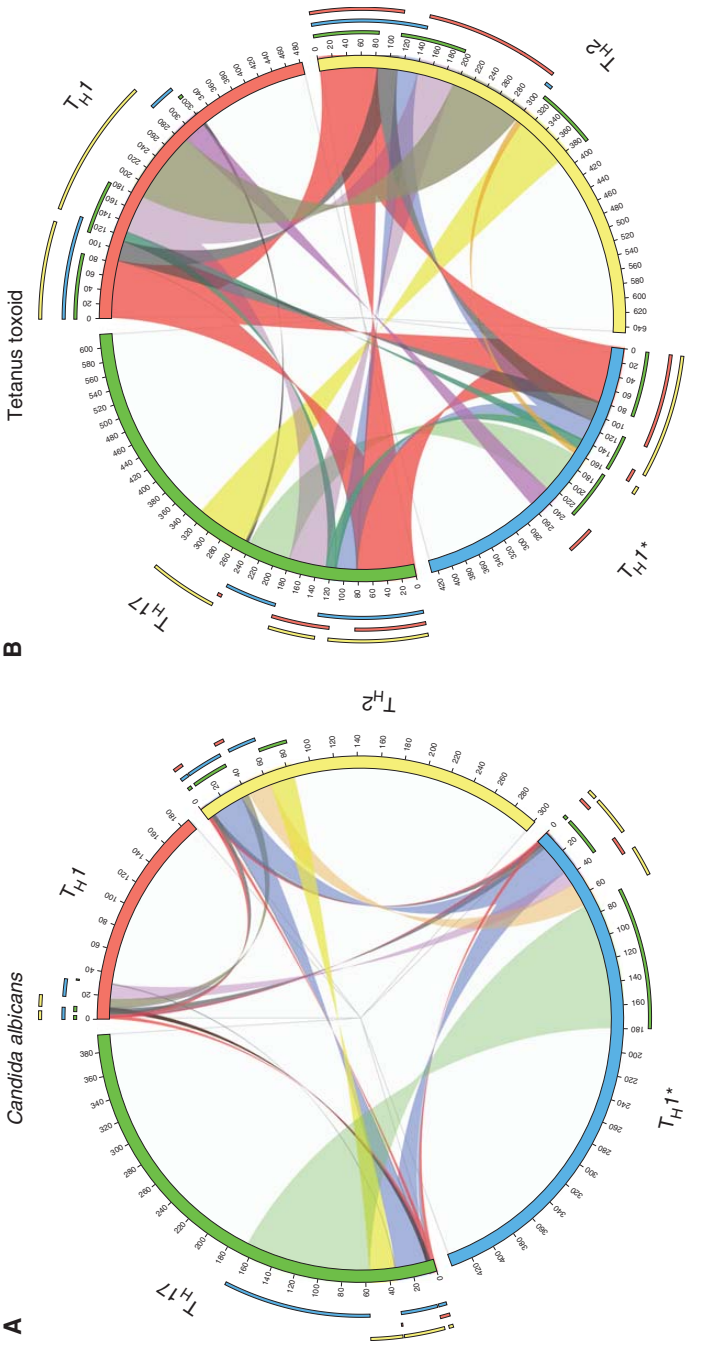


**Figure 2.** Graphical representation of the extent of sharing among T-cell antigen receptor (TCR) clonotypes of memory $T_H1$ (red), $T_H2$ (yellow), $T_H1^*$ (blue), and $T_H17$ (green) cells primed by *Candida albicans* (*A*), or tetanus toxoid (*B*). Outer rings and connecting lines identify clonotypes shared between two or more T-cell subsets. Data are representative of single donors. (From Becattini et al. 2015; modified, with permission.)



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ity, TCR sequence, and phenotype from single T cells will likely unravel even further degrees of interclonal and intraclonal heterogeneity in the T-cell response to microbial pathogens and vaccines (Han et al. 2014). Resolving the behavior of T-cell populations at the level of individual cells and in the context of protective or pathological immune responses is a major challenge that holds promise for a better understanding of the immune system and for immune interventions.

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# Do Memory CD4 T Cells Keep Their Cell-Type Programming: Plasticity versus Fate Commitment?

## Complexities of Interpretation due to the Heterogeneity of Memory CD4 T Cells, Including T Follicular Helper Cells

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Plasticity is the ability of a cell type to convert to another cell type. There are multiple effector CD4 T-cell subtypes, including $T_H1$, $T_H2$, $T_H17$, $T_H1^*$, CD4 CTL, $T_H9$, and $T_{FH}$ cells. It is commonly thought that a CD4 T cell can readily show full plasticity—full conversion from one differentiated cell—and this propensity to plasticity is possessed by memory CD4 T cells. However, there remains no direct demonstration of in vivo–generated resting memory CD4 T-cell conversion to a different subtype on secondary antigen challenge in vivo in an intact animal at the single-cell level. What has been clearly shown is that CD4 T cells possess extraordinary capacity for phenotypic heterogeneity, but that is a distinct property from plasticity. Heterogeneity is diversity of the resting memory CD4 T-cell population, not conversion of a single differentiated cell into another subtype. Apparently, plasticity at the population level can be accomplished by either mechanism, as heterogeneity of CD4 T-cell subpopulations could affect large shifts in subtype distribution at the overall population level via differential exponential expansion and death.

OUTLINE



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Here, plasticity is defined as the conversion of a single cell possessing a well-characterized CD4 T-cell type into a cell no longer possessing that phenotype and instead possessing a different well-characterized CD4 T-cell phenotype. For example, conversion of a memory $T_H1$ cell (T-bet$^+$IFN-$\gamma^+$CXCR5$^-$Bcl6$^-$) into a $T_{FH}$ cell (Bcl6$^+$CXCR5$^+$T-bet$^-$IFN-$\gamma^-$) would be plasticity. Separately, heterogeneity within a well-characterized CD4 T-cell population is defined here as a collection of varied phenotypes ($<$100% of the cell population) linked by a shared core phenotype. For example, heterogeneity among $T_H1$ cells can be observed by flow cytometry or mass cytometry by defining $T_H1$ cells as T-bet$^+$IFN-$\gamma^+$ cells and then observing fractions of the population expressing tumor necrosis factor (TNF), or interleukin (IL)-2, or Blimp1, or IL-10, or Eomes, etc. As another example, heterogeneity among $T_H2$ cells can be observed by flow cytometry or mass cytometry by defining $T_H2$ cells as GATA3$^{hi}$ cells and then observing fractions of the population expressing IL-5, IL-4, IL-13, CRT$_H$2, CCR4, or IL-10, etc. As another example, heterogeneity among germinal center (GC) $T_{FH}$ cells can be observed by flow cytometry or mass cytometry by defining GC $T_{FH}$ cells as Bcl6$^+$CXCR5$^+$ cells and then observing fractions of the population expressing CXCL13, IL-21, IL-4, or CXCR3, etc. (Crotty 2014; Vinuesa et al. 2016). Heterogeneity at the whole population level further includes the range of differentiated CD4 T-cell subtypes present, including $T_H1$, $T_H2$, $T_H17$, $T_H1^*$, CD4 CTL, $T_H9$, and $T_{FH}$ cells, and perhaps even some form of "unbiased" $T_H0$-type cells. Both plasticity and heterogeneity must be described based on analyses at the single-cell level.

Reports of T-cell program plasticity are unconvincing when the data are population-level changes in phenotypes. Such results can easily be the outcome of outgrowth of a minor cell population to become the dominant cell population, or vice versa, particularly given the exponential proliferation that T cells are capable of. Also unconvincing are the relevance of reports of cell program plasticity for which the central experiments are cell transfers into new hosts, particularly new hosts that are severely immunocompromised (e.g., T-cell-deficient mice). Such experiments show that CD4 T-cell plasticity can occur under extreme conditions, but the experiments have no demonstrated relevance to what CD4 T cells actually do or experience in an intact animal. In contrast, if transferred cells do maintain stability, those results are more credible, because they show stability of cell identity even when exposed to nonphysiological stresses. Apparent plasticity of differentiated CD4 T cells in vitro is generally not convincing, both because the in vitro experiments lack demonstrated in vivo relevance and because the experiments are performed at the cell population level, masking the impact of outgrowth of minor cell populations. The strictest criterion for demonstration of plasticity is the use of a lineage marker reporter transgenic mouse, tracking, over time, cells marked irreversibly. Such an experiment directly establishes the transcriptional history of a given cell. Many lineage-tracking experiments have been performed on nTregs, making use of Foxp3-IRES-GFP/YFP/RFP-Cre-based designs (Rubtsov et al. 2008, 2010; Zhou et al. 2009; Miyao et al. 2012). The central conclusions from the two later studies with more sophisticated modified *Foxp3* gene reporter constructs was that Foxp3$^+$ nTregs are very stable, with almost no plasticity (Rubtsov et al. 2010; Miyao et al. 2012). In contrast, substantial gene-expression heterogeneity could be observed in conditions of stress and while still maintaining core Foxp3$^+$ nTreg programming. Still, the stability conclusions drawn from such studies are not necessarily directly transferrable for antigen-specific CD4 T-cell responses and CD4 T-cell memory, because nTregs develop their initial programming during thymic development.

## STABILITY DURING A PRIMARY RESPONSE

There are no lineage marker reporter mouse studies showing plasticity of $T_H1$, $T_H2$, $T_H17$, or $T_{FH}$ cells during a primary immune response in an intact animal. Thus, excluding thymic-derived Tregs, there is no definitive evidence of physiologically relevant CD4 T-cell plasticity during a primary immune response. Cell-trans-



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fer experiments have attempted to address stability or plasticity of antigen-specific CD4 T cells during a primary immune response. We observed that $T_{FH}$ and $T_H1$ cells during a viral infection establish largely irreversible cell fates by 72 h postinfection, based on cell transfers of virus-specific $T_H1$ or $T_{FH}$ cells from virally infected mice into time-matched virally infected mice (Choi et al. 2013). Similar pronounced cell-fate commitment results were independently reported using a protein immunization and an RFP-Bcl6 reporter mouse strain when transferring $CXCR5^-Bcl6^-$ or $CXCR5^+Bcl6^+$ cells at day 7 postinfection (Liu et al. 2012). Plasticity of $T_H1$ and $T_H2$ cells to become $T_{FH}$ cells has been reported; however, those experiments used in vitro–generated $T_H1$ and $T_H2$ cells transferred into mice (Liu et al. 2012) or in vitro polarized cells then repolarized under different in vitro conditions (Lu et al. 2011). It is almost certainly the case that there is a window of time early during effector CD4 T-cell differentiation in a primary immune response when a given CD4 T cell possesses pluripotency, simultaneously expresses lineage-defining transcription factors (e.g., Bcl6 and T-bet and RORγt) (Nakayamada et al. 2011; Oestreich et al. 2012), and maintains the capacity to respond to different extrinsic signals and subsequently commit to one differentiated cell type (e.g., $T_{FH}$ or $T_H1$ or $T_H17$) (DuPage and Bluestone 2016). Thus, simple questions regarding durable stability versus plasticity must be assessed after that point, which is nontrivial to accomplish.

## STABILITY DURING TRANSITION FROM EFFECTOR CELL TO MEMORY CELL

The transition from an effector CD4 T cell to a central memory CD4 T cell appears to also be a transition from a cell with a highly polarized gene-expression program to a cell with a less polarized gene-expression program. This may be key to understanding the apparent plasticity of memory CD4 T cells, discussed below.

Based on single-cell transfer studies in mouse model systems, most CD4 T-cell clones are capable of generating memory cells (Tubo et al. 2016), and a given individual CD4 T-cell clone can differentiate into multiple different CD4 T-cell types (e.g., $T_{FH}$ and $T_H1$) as they divide during a primary immune response (Tubo et al. 2013). Furthermore, those effector cells can then develop into memory $T_{FH}$ and $T_H1$ cells in frequencies comparable with the frequencies of $T_{FH}$ and $T_H1$ cells generated by that clone during the effector phase of the CD4 T-cell response (Tubo et al. 2016). Human T-cell receptor (TCR) sequencing clonotype analysis of antigen-specific human memory CD4 T cells has shown that a given TCR sequence can be found in $T_H1$, $T_H2$, and $T_H17$ antigen-specific central memory cells (Becattini et al. 2015), consistent with the mouse model observation.

During a primary immune response, it has been observed that $T_{FH}$ cells can have gene expression of other T-cell differentiation programs. In the context of a mouse with an acute lymphocytic choriomeningitis virus (LCMV) infection, the mantle $T_{FH}$ cells ($mT_{FH}$, outside of GCs) and GC $T_{FH}$ cells express T-bet and interferon γ (IFN-γ) at substantially higher amounts than naïve CD4 T cells (Johnston et al. 2009; Yusuf et al. 2010; Ray et al. 2015). In our first paper on $T_{FH}$ cells, we stated "it is notable that T-bet and IFN-γ were still expressed in the $T_{FH}$ in vivo, although at lower levels than in $T_H1$/non-$T_{FH}$ LCMV-specific CD4 T cells. These observations are consistent with a model in which $T_{FH}$ cells follow their own differentiation pathway but are not an isolated lineage and can show partial characteristics of $T_H1/T_H2$ polarization depending on environmental conditions." Similar observations have been made for simian immunodeficiency virus (SIV) infection of rhesus macaques (Iyer et al. 2015). Given that both LCMV and SIV infections are extreme $T_H1$-biased immune responses, the presence of $T_H1$ gene expression by $T_{FH}$ cells in LCMV, and SIV immune response may represent uncommon exceptions. In support of that concept, human tonsillar GC $T_{FH}$ cells expressing $T_H1$, $T_H2$, or $T_H17$ cytokines are rarely observed (Ma et al. 2009; Yu et al. 2009; Dan et al. 2016; Havenar-Daughton et al. 2016). For example, $<1\%$ of GC $T_{FH}$ cells produce IL-17 (Yu et al. 2009; Wong et al. 2015; Dan et al. 2016). Mouse GC $T_{FH}$ cells rarely produce IL-



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13, even under very strong $T_H2$ polarizing helminth infection conditions (Liang et al. 2012), or house dust mite sensitization (Ballesteros-Tato et al. 2016) ($T_{FH}$ cells normally express IL-4 as part of canonical $T_{FH}$ programming, distinct and independent of $T_H2$ programming, and thus $T_{FH}$ expression of IL-4 is not an indication of any $T_H2$ gene programming [Crotty 2011]). Furthermore, Bcl6 represses many $T_H1$, $T_H2$, and $T_H17$ genes and can prevent $T_H1$ differentiation (Johnston et al. 2009; Oestreich et al. 2012; Hatzi et al. 2015). A counterargument can now be made that cytokine expression by intracellular cytokine staining is insufficiently sensitive to determine whether a GC $T_{FH}$ cell may possess $T_H1$, or $T_H2$, or $T_H17$ gene expression, because GC $T_{FH}$ cells are intrinsically stingy cytokine producers, and intracellular cytokine staining missed ~98% of human or macaque antigen-specific GC $T_{FH}$ cells (Dan et al. 2016; Havenar-Daughton et al. 2016). Thus, single-cell RNAseq of GC $T_{FH}$ cells may be required to better understand whether GC $T_{FH}$ cells with partial $T_H1$, $T_H2$, or $T_H17$ heterogeneity characteristics are common or rare.

Considering the process from the opposite direction, it is clear that human memory $T_{FH}$ cells can show certain phenotypic markers commonly associated with $T_H1$, $T_H2$, or $T_H17$ cells (discussed more in the next section). What is the ontogeny of those cells? One possibility is that $T_{FH}$ cells can be imprinted with a fractional amount of $T_H1$ gene programming by an antigen-presenting cell during the early stages of T-cell priming in response to a $T_H1$ pathogen, and, although that gene-expression program is efficiently squelched by Bcl6 in the effector $mT_{FH}$ and GC $T_{FH}$ progeny of that cell, a partial $T_H1$ program remains imprinted (Fig. 1). As a GC $T_{FH}$ cell transitions into a memory cell, it loses expression of Bcl6 protein (Kitano et al. 2011; Liu et al. 2012; Choi et al. 2013; Hale et al. 2013; Locci et al. 2013; Ise et al. 2014), thus derepressing a range of genes, including *Ccr7* and *Il7ra* (Fig. 1) (Kitano et al. 2011; Choi et al. 2013). One possibility is that the loss of Bcl6 protein as the GC $T_{FH}$ cell transitions into a memory $T_{FH}$ cell can allow a partial $T_H1$ program imprinted at the time of T-cell priming to then become derepressed in some cells in the absence of Bcl6 protein, resulting in the central memory $T_{FH}$ cell acquiring a partially mixed $T_{FH}/T_H1$ phenotype (Fig. 1, model 1). Such an event may be considered "imprinted partial plasticity" because the phenotype of the cell would be dependent on the initial signals it received during T-cell priming, even if the gene-expression program was kept silent for long periods of time. If the cell were to subsequently become a $T_H1$ cell and lose $T_{FH}$ characteristics, that would be "imprinted full plasticity." This concept has not been directly tested.

A second possibility (model 2) is that a GC $T_{FH}$ cell that does not have any $T_H1$, $T_H2$, or $T_H17$ gene expression or imprinting may be induced to activate a partial $T_H1$, $T_H2$, or $T_H17$ gene-expression program if there are $T_H1$, $T_H2$, or $T_H17$ differentiation inductive signals still present in the environment when the GC $T_{FH}$ cell is transitioning to become a memory cell and losing Bcl6 protein expression (Fig. 1). Such an event would be CD4 T-cell plasticity, termed "de novo partial plasticity" here, to distinguish it from imprinted plasticity.

Alternatively, a substantial proportion of memory $T_{FH}$ cells may be generated very early during an immune response derived from mantle $T_{FH}$ cells ($mT_{FH}$) without going through a GC $T_{FH}$ cell stage. Evidence for such a pathway comes from studies of *Sh2d1a*$^{-/-}$ mice and humans, which have CD4 T cells that can differentiate into $mT_{FH}$ cells but not GC $T_{FH}$ cells and have evidence of $T_{FH}$ cell memory (He et al. 2013). If memory $T_{FH}$ cells are generated via such a pathway in immunocompetent mice and humans, it is plausible that memory $T_{FH}$ cells derived from $mT_{FH}$ cells may be less polarized than memory $T_{FH}$ cells derived from GC $T_{FH}$ cells, with less fixed-fate programming, and therefore may be more likely to have mixed attributes of $T_{FH}$ and $T_H1$ or other programs (model 3). As is the case for models 1 and 2, this concept has also not been directly tested.

Given the observations and models described above, the presence of $CXCR3^+$ $CXCR5^+$ memory CD4 T cells in human peripheral blood is consistent with differentiation models based on heterogeneity, imprinted



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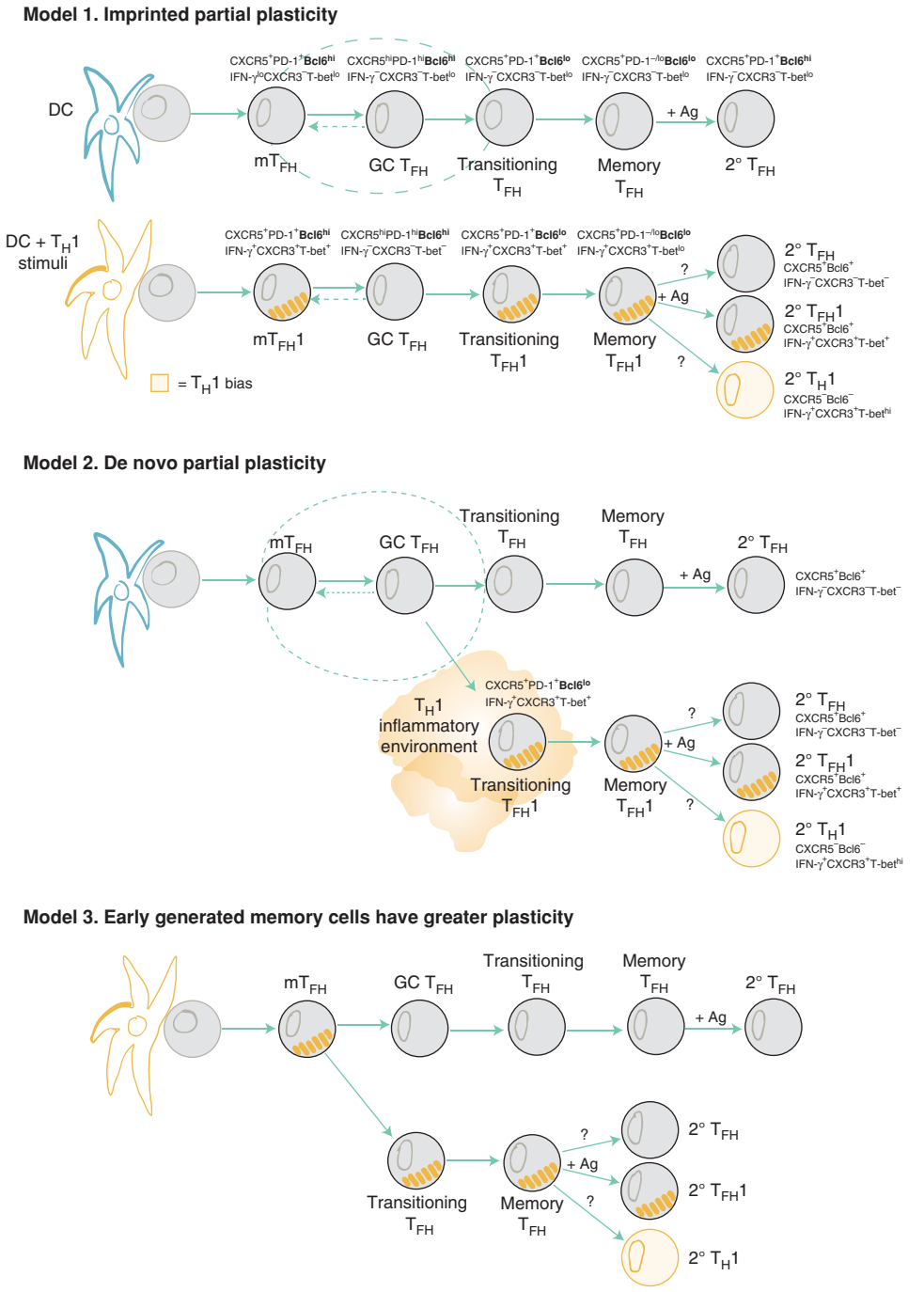


**Figure 1.** Three models of the development of heterogeneous or plastic CD4 T-cell memory. Each model is discussed in the main text.



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partial plasticity, or de novo partial plasticity. CXCR3 is expressed by $T_H1$ cells and is a direct target of T-bet, and the CXCR3$^+$ CXCR5$^+$ memory CD4 T cells in human peripheral blood cells are almost all capable of producing IFN-γ on stimulation (Morita et al. 2011; Bentebibel et al. 2013; Locci et al. 2013; Obeng-Adjei et al. 2015). Thus, CXCR3$^+$ CXCR5$^+$ memory CD4 T cells could have potentially derived from CXCR3$^+$ GC $T_{FH}$ cells or $mT_{FH}$ cells (Iyer et al. 2015), or CXCR3$^-$ GC $T_{FH}$ cells or $mT_{FH}$ cells that were exposed to a $T_H1$ environment while transitioning to memory $T_{FH}$ cells.

CD4 T-cell biology usually does not fit tidy single pathway models; heterogeneity of phenotypes and differentiation patterns are common, as this is likely important to confound pathogen immune evasion strategies (Crotty 2012). Thus, a new report is surprising, but perhaps should not have been. Development of $T_H2$ cells (IL-5$^+$ IL-13$^+$ CXCR5$^-$) in the lungs in response to a second exposure to house dust mite antigens was observed to be dependent on effector $T_{FH}$ cells, with multiple lines of evidence pointing to differentiation of GC $T_{FH}$ cells (CXCR5$^+$PD-1$^{hi}$ IL-21$^+$) into $T_H2$ cells (Ballesteros-Tato et al. 2016). These appeared to be fully differentiated active GC $T_{FH}$ cells, to the best of the ability of the authors to sort a pure cell population, with the previously stated caveats. In contrast, a different group, using a different IL-21 reporter mouse, did not observe $T_{FH}$ cells to be precursors to $T_H2$ cells (IL-33R$^+$ IL-5$^+$ IL-13$^+$) in a similar house dust mite model (Coquet et al. 2015), but they did not gate on CXCR5$^+$ cells for the cell sorts. The two groups also transferred cells at different times after the primary antigen exposure, which may result in tracking cells at different points of cell-fate commitment. When $T_{FH} \rightarrow T_H2$ plasticity occurred, the $T_{FH}$ cells were taken 6 d after the primary immunizations (Ballesteros-Tato et al. 2016). In neither study were resting memory CD4 T cells used (cells without activation marker expression taken at $>$30 d after the last antigen exposure). Lineage-tracking models that do not depend on cell transfers are likely to be the only means of resolving such disparate observations.

## MEMORY CD4 T-CELL PHENOTYPE HETEROGENEITY AND STABILITY DURING RESTING MEMORY

Resting memory CD4 T cells appear to be largely stable over time by major lineage-defining phenotypic markers, as shown for antigen-specific $T_{FH}$, $T_H1$, and $T_H2$ cells in mouse models (Harrington et al. 2008; Hale et al. 2013; Hondowicz et al. 2016; Tubo et al. 2016). Human data support the same conclusion but the antigen-specific data are limited (Locci et al. 2013; Bancroft et al. 2016; Da Silva Antunes et al. 2017). No longitudinal data on antigen-specific resting memory CD4 T-cell phenotypes from individual human donors are available at single-cell flow-cytometric resolution, which is much needed to demonstrate memory CD4 T-cell subset stability.

$T_H17$ cells may be an exception to memory. There remains little evidence showing clear demonstration of in vivo–generated $T_H17$ memory cells in mice. $T_H17$ memory was absent in intact mice in one longitudinal antigen-specific CD4 T-cell study (Pepper et al. 2010). A later paper reported $T_H17$ memory, but it depended on transfer of in vitro–generated $T_H17$ cells (Muranski et al. 2011). Although an *Il17a* lineage marking reporter mouse has been available for many years (Hirota et al. 2011), there is a lack of publications on in vivo–generated $T_H17$ memory. In humans, presence of antigen-experienced $T_H17$ cells to *Candida albicans* has been clearly demonstrated (Zielinski et al. 2012); however, recurrent or continual exposure was not excluded, and a resting memory $T_H17$ phenotype (e.g., Ki67$^-$) was not shown for *C. albicans*–specific cells. Thus, although the existence of resting stable memory $T_H17$ cells seems biologically reasonable and there is indirectly supportive literature (Lindenstrøm et al. 2012; McGeachy 2013), data showing antigen-specific resting memory $T_H17$ cells with single-cell analysis are currently quite limited.

Although there has been evidence of heterogeneity within CD4 T-cell subsets, going back to early descriptions of $T_H1$ and $T_H2$ cells (e.g., $T_H2$ cells producing some or all of IL-4, IL-5,



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IL-13, and IL-10), the vastness of the dimensional space of memory T-cell phenotypic heterogeneity was first made evident by the human memory CD8 T-cell mass spectrometry study of Newell and Davis (Newell et al. 2012). There was such phenotypic variety of memory CD8 T cells to influenza and cytomegalovirus (CMV) that the authors did not even attempt to put a number on the total range of memory CD8 T-cell phenotypes observed; instead, the diversity was best calculated as large spaces of phenotypic variation in three-dimensional principal component analysis (PCA) plots (Newell et al. 2012). Subsequent mass spectrometry analysis of human CD4 T cells has shown even more heterogeneity (Wong et al. 2015), consistent with the diversity of $T_H1$, $T_H2$, $T_H17$, $T_{FH}$, $T_H9$, $T_H1^*$, CD4 CTL, and iTreg biology. Importantly, phenotypic diversity in human memory CD4 T cells is seen even at the level of individual TCR clonotypes (Becattini et al. 2015). One example of heterogeneity is the presence or absence of PD-1 expression by resting memory $T_{FH}$ cells (Locci et al. 2013). Heterogeneity is clearly present among chemokine receptor expression by memory CD4 T cells. A population of $CCR6^+$ $CXCR5^+$ resting memory CD4 T cells is present in human peripheral blood, and it has been suggested those cells represent "$T_{FH}17$" memory cells (Morita et al. 2011). However, CCR6 expression is not specific to $T_H17$ cells and does not correlate well with $T_H17$ programming in many cases. One report found no IL-17a expression by stimulated $CCR6^+$ $CXCR5^+$ memory CD4 cells in single-cell analysis (Wong et al. 2015). Therefore, most CCR6 expression by memory $T_{FH}$ cells may be unrelated to $T_H17$ biology and simply reflective of preferential chemotaxis needs. A similar situation exists for "$T_{FH}2$" memory cells. "$T_{FH}2$" memory CD4 T cells in human peripheral blood are commonly defined only on the basis of negative markers ($CXCR3^-CCR6^-$) (Morita et al. 2011; Jacquemin et al. 2015), based on the assumption that all $T_{FH}$ memory cells must have a $T_H1$, $T_H2$, or $T_H17$ bias (which has certainly not been demonstrated at the single-cell level for GC $T_{FH}$ cells or resting memory $T_{FH}$ cells); thus, the concept of $T_{FH}2$ cells remains poorly defined. Given that $T_{FH}$ cells produce IL-4 in a $T_{FH}$-specific manner (independent of the $T_H2$ program) (Crotty 2011), identification of a $T_{FH}2$ cell requires demonstration of a resting memory $CXCR5^+$ CD4 T cell capable of producing IL-5 and/or IL-13 at the single-cell level—a criterion not reached in the current literature. Such cells may exist, but they have not been shown directly, and they may be a minor fraction of the $CXCR3^-CCR6^-$ memory $T_{FH}$ cells. There is clearly vast phenotypic heterogeneity among resting memory CD4 T cells, including resting memory $T_{FH}$ cells. However, at the level of cytokine production, it is still unknown how rare memory $T_{FH}$ cells with cytokine production attributes of other CD4 T-cell subsets are, except for overlap between $T_H1$ and $T_{FH}$ programs in memory CD4 T cells, which has been clearly shown by multiple groups.

## PLASTICITY WHEN MEMORY CD4 T CELLS ARE RECALLED?

A first study using an IL-21-GFP reporter mouse observed extensive plasticity of IL-21-$GFP^+$ $CXCR5^+$ $T_{FH}$ or IL-21-$GFP^-$ $CXCR5^+$ cells after transfer into new hosts, with $<50\%$ of the memory cells maintaining CXCR5 expression, and a majority of the cells observed after recall by influenza infection were $CXCR5^-$ (Lüthje et al. 2012). A newer IL-21 fluorescent protein reporter mouse model observed robust stability of IL-$21^+$ $CXCR5^+$ $T_{FH}$ CD4 T cells after transfers into new hosts, but did not test memory time points (Weinstein et al. 2016). In the context of an acute LCMV infection, memory CD4 T cells appear to largely maintain their $T_H1$ or $T_{FH}$ programming upon 2° response. TCR transgenic memory CD4 T cells with a resting $T_H1$ phenotype all became effector $T_H1$ cells (T-$bet^{hi}Bcl6^{lo}CXCR5^-$ Granzyme $B^+$) when transferred into a new host that was then infected with LCMV. In the same study, the major of memory CD4 T cells with a resting $T_{FH}$ phenotype became effector $T_{FH}$ and GC $T_{FH}$ cells on rechallenge (T-$bet^{lo}Bcl6^+CXCR5^+$Granzyme $B^-$). A fraction of the memory $T_{FH}$ cells did lose CXCR5 in



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the 2° response, but because those experiments depended on cell transfers, it is unknown whether all of the memory $T_{FH}$ cells would have maintained their $T_{FH}$ program on 2° challenge under physiological conditions in an intact animal where they were allowed to maintain their normal localization. It is, of course, also formally possible that the memory cells would have showed even more plasticity if studying in an intact host. Another longstanding challenge with sorted cells that have high proliferative capacity such as lymphocytes is that even a 1% $CXCR5^-$ contamination of sorted cells could subsequently expand extensively during the exponential proliferation following 2° challenge, thus confounding cell-fate interpretations of cell-transfer experiments.

Resting central memory CD4 T cells show less polarized features than actively responding effector cells. Resting central memory $T_H1$ or $T_{FH}$ cells have less active transcription and protein expression of many signature features of activated $T_H1$ cells or activated GC $T_{FH}$ cells. For example, central memory $T_H1$ cells express low levels of T-bet compared with effector cells, and resting memory $T_{FH}$ cells express Bcl6 but at levels indistinguishable from other central memory cells. Intuitively, one expects such cells to be prone to plasticity. Programmed chromatin modifications may prevent plasticity. Memory $T_{FH}$ cells rapidly up-regulate Bcl6 in vivo on restimulation (Ise et al. 2014). It is unknown whether memory $T_{FH}$ cells with mixed $T_{FH}$–$T_H1$ phenotypes ($CXCR3^+CXCR5^+$) differentiate into $T_{FH}$ ($CXCR3^-CXCR5^+Bcl6^+$ $T\text{-}bet^-$), $T_{FH}1$ ($CXCR3^+CXCR5^+Bcl6^+T\text{-}bet^+$), and/or conventional $T_H1$ cells ($CXCR3^+$ $CXCR5^-Bcl6^-T\text{-}bet^+$) in vivo in an intact mouse or human (Fig. 1). Evidence of plasticity was not observed in humans at the level of the overall CD4 T-cell response to pertussis, wherein the whole-cell pertussis vaccine and the acellular pertussis vaccine elicit predominantly $T_H1$ and $T_H2$ polarizing responses, respectively, and reimmunization with the acellular pertussis vaccine elicits a CD4 T-cell recall response with the same $T_H1$ or $T_H2$ characteristics for whichever vaccine the individual was initially immunized (Bancroft et al. 2016).

## CONCLUDING REMARKS

There remains no direct demonstration of in vivo–generated resting memory CD4 T-cell conversion to a different subtype on secondary antigen challenge in vivo in an intact animal at the single-cell level. Lineage tracing experiments are needed to directly test whether plasticity occurs and, more importantly, how common or rare the process is under physiological conditions. Unfortunately, proper lineage-tracking genetic models are difficult to generate for $T_{FH}$, $T_H17$, and $T_H2$ cells. The lineage-defining transcription factors for $T_{FH}$, $T_H17$, and $T_H2$ cells are Bcl6, RORγT, and GATA3. GATA3 is constitutively expressed by all CD4 T cells; $T_H2$ cells are defined by high GATA3 expression. Thus, a conventional GATA3 lineage reporter would mark all T cells. Therefore, development of a high-fidelity $T_H2$ lineage genetic marker is a difficult challenge. A successful $T_H2$ lineage genetic marker would probably need to depend on transcription from a gene or locus other than GATA3. Bcl6 is highly expressed by thymocytes, and thus a Bcl6 lineage reporter mouse constructed based on standard designs would be expected to mark most T cells. RORγt is also expressed by thymocytes and, thus, an RORγt lineage reporter mouse based on standard designs would be expected to mark most T cells. Therefore, a successful $T_{FH}$ or $T_H17$ lineage genetic marker would probably need to depend on transcription from a gene or locus other than GATA3; IL-17a is one candidate for $T_H17$ cells (Hirota et al. 2011). CXCR5 may be a good candidate for $T_{FH}$ cells. As for $T_H1$ cells, a T-bet lineage reporter should be useful, but a key caveat is that transient expression of T-bet early on by a naïve CD4 T cell after activation is common and may have no influence on the future history of the cell. The same caveat applies for all constitutively active lineage marker systems, and contributed to controversy over the ontogeny of "exTregs." Transient expression of Foxp3 by some cells may result in erroneous conclusions on the basis of very transient Cre expression (Rubtsov et al. 2008; Zhou et al. 2009; Miyao et al. 2012). This concern may be



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avoided by using estrogen-responsive Cre protein (Rubtsov et al. 2010).

As an alternative to lineage-tracking genetic markers, single-cell transfers into infection-matched hosts may be the best test of stability versus plasticity in a recall response. Such experiments would need to show that transferred cells (not using single-cell transfers) would localize in the new host to the same regions of lymph node (LN) and spleen as untransferred antigen-specific resting memory CD4 T cells of that subtype (e.g., proper microanatomical localization of memory $T_{FH}$ cells posttransfer).

In the end, it is unknown whether the appearance of plasticity by memory CD4 T cells at the population level is predominantly because of heterogeneity and outgrowth of subpopulations or predominantly attributable to plasticity.

It remains possible that true memory CD4 T-cell plasticity may be primarily of interest for purely academic reasons, as a memory recall response with apparent plasticity at the whole-cell population level could presumably be accomplished via rapid outgrown of a very minor population of memory cells. Because essentially all in vivo CD4 T-cell responses involve a mixture of subtypes ($T_H1$, $T_H2$, $T_H$ CTL, and $T_{FH}$, for example), that scenario is plausible.

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