Published in final edited form as:

Immunol Rev. 2013 March ; 252(1): 24–40. doi:10.1111/imr.12037.

Helper T Cell Identity and Evolution of Differential Transcriptomes and Epigenomes

Golnaz Vahedi^{1,*}, Amanda Poholek^{1,*}, Timothy W. Hand², Arian Laurence¹, Yuka Kann¹, John J. O'Shea¹, and Kiyoshi Hirahara¹

¹Lymphocyte Cell Biology Section, Molecular Immunology and Inflammation Branch, National Institutes of Arthritis, and Musculoskeletal and Skin Diseases, Bethesda, MD, USA

²Laboratory of parasitic diseases, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA

Summary

CD4⁺ T cells are critical for the elimination of an immense array of microbial pathogens. Among the ways they accomplish this task is to generate progeny with specialized, characteristic patterns of gene expression. From this perspective, helper cells can be viewed as pluripotent precursors that adopt distinct cell fates. Although there are aspects of helper cell differentiation that can be modeled as a classic cell fate commitment, CD4⁺ T cells also maintain considerable flexibility in their transcriptional program. This makes sense in terms of host defense but raises the question of how these remarkable cells balance both these requirements, a high degree of specific gene expression and the capacity for plasticity. In this review, we discuss recent advances in our understanding of CD4⁺ T-cell specification, focusing on how genomic perspectives have influenced our views of these processes. The relative contributions of sensors of the cytokine milieu, especially the signal transducer and activator of transcription (STAT) family transcription factors, 'master regulators', and other transcription factors are considered as they relate to the helper cell transcriptome and epigenome.

Keywords

T-cell plasticity; STAT; epigenetics; histone modification; T-cell differentiation; enhancers; master regulator

Introduction

CD4⁺ T cells are critical components in orchestrating immune responses and facilitating elimination of offending microorganisms. Conversely though, CD4⁺ T cells are also major players in mediating autoimmune and allergic diseases. In response to infectious agents and other stimuli, naive CD4⁺ T cells differentiate into distinct cellular subsets that express a unique set of cytokines. The selective production of cytokines is a major means by which CD4⁺ T cells exert their immunoregulatory effect. The restricted programs of gene expression have led to the view of differentiated T cells as distinct lineages. However, CD4⁺ T cells also have an impressive capacity to modulate their responses, displaying considerable functional plasticity. In this review, we discuss the extrinsic and intrinsic factors that control phenotypic specification and flexibility of CD4⁺ T cells. We discuss how

Correspondence to: John J. O'Shea 10 Center Dr., Bldg10, Room 13C103 Bethesda, MD 20892-1930, USA Tel.: +1 301 435 7657
Fax: +1 301 480 6372 osheajo@mail.nih.gov.

*These authors contributed equally to the writing of this article.

these factors impact gene expression and the epigenetic landscape of these cells. Before reviewing these topics in detail, it is worthwhile considering the general issue of cellular identity and what we have learned about the mechanisms responsible. This is particularly relevant as technical advances are rapidly changing how we view the factors that control cellular phenotypes.

Cell identity: a primer on transcriptomic and epigenomic views

Understanding how unique cell identities are established has intrigued biologists for several centuries. The view that different cells express different genes and this differential expression defines cellular identity has long been appreciated. The completion of various genome projects allows comprehensive assessment of selective gene expression. Thus, surveying the transcriptome of distinct subsets of cells is now commonplace.

Selective gene expression is mediated in part by *trans*-acting DNA binding proteins, especially so-called transcription factors, which recognize specific DNA sequences. Thanks to the completion of the human genome project, we now know that there are more than 1000 of these factors. Among various classes of transcription factors, some are broadly expressed and activated in response to changes in cellular environment or by key surface receptors. Other factors are induced in response to cellular stimulation. We do not understand the function of many transcription factors, but one thing is clear - complex regulatory networks work in concert to promote expression of lineage-specific genes and repress genes of opposing fates.

However, transcription factors are not the only factors that dictate cell-type-specific transcriptomes. Distinctive programs of gene expression are also regulated by the accessibility of these genes, which can authorize or deny transcription factor functionality. Such regulated access of the genetic code is a major means by which a single genome can give rise to many morphologically distinct cell types. This leads to the notion of 'epigenetic' regulation, which has been classically defined as heritable phenotypic changes not due to changes in DNA sequence. Until recently though, what the epigenome meant in terms of cell biology and biochemistry has been unclear and an understanding of how intrinsic and extrinsic specifying factors impact epigenetics remained elusive. However, advances in this area are now accumulating at an astonishing pace.

The accessibility of genes residing in active (euchromatin) and silenced (heterochromatin) configurations is a well-accepted view of epigenetic regulation. Variation in the packaging of DNA into chromatin can provide an opportunity or an obstacle for transcription factors to execute their functions. DNA is wrapped into nucleosomes that consist of histone protein octamers resembling 'beads-on-a-string'. The histone components of the nucleosome are associated with an array of post-translational modifications. For example, histone 3 lysine 4 trimethylation (H3K4me3) is associated with active promoters and histone 3 lysine 36 trimethylation (H3K36me3) is indicative of active transcription of genes. In contrast, histone 3 lysine 27 trimethylation (H3K27me3) and histone 3 lysine 9 trimethylation (H3K9me3) are marks of silenced genes. Mechanistically, the term 'epigenetic' has come to encompass modifications of the histone molecules, DNA methylation, and regulatory RNAs (microRNAs and long noncoding RNAs). The current view is that the cell-type-specific status of the chromatin landscape or epigenome defines targets of transcription factor proteins and consequently allows gene expression.

While biological differences between cell types emerge from differences in gene expression patterns, complexity is not a function of increased numbers of genes (1). It is now evident that a correspondingly complex set of regulatory information in the genome is responsible for cellular diversity. The completion of the human genome project revealed that only 2% of

the genome represents protein coding genes, but recent findings from the ENCODE project has revealed that the remainder of the genome is not 'junk'. On the contrary, the vast majority (80%) of the non-coding part of the human genome is active. Amazingly, thanks to the ENCODE project, it appears that three-quarters of the human genome is capable of being transcribed (2)! Thus, in considering cell identity, one must not be limited by a gene-centric view of the genome. Indeed, ENCODE makes one reconsider what it means to be a gene. Regulation of phenotype is not just about encoding protein; enormous stretches of the genome consist of instructions or regulatory elements that supervise protein-coding genes. As a result, understanding cell identity requires the understanding of critical switches and control elements, which may appear to be far removed from genes.

Among these regulatory elements, 'enhancers' play a particularly fundamental role in gene regulation. An emerging perspective is that enhancers are the critical determinants of cell identity (3). Enhancers can activate transcription regardless of the element's location or orientation relative to the promoter of their target genes. This is because seemingly disparate parts of the genome are nucleated into regulatory hubs. As will become evident, the complex, tissue-specific expression of genes, such as those required for immune cell function, often require large segments of the genome. Prediction of enhancer elements on a broad scale has been challenging however. Investigation of DNA sequences including analysis of evolutionary conservation or transcription factor binding motifs has been employed (1, 4-6). The limitation of these analyses of evolutionary conservation is that it fails to predict where and when the regulatory element is active. Transcription factor binding site motifs are also degenerate. Thus, both the approaches have enjoyed limited success.

More recently, various features of chromatin have been exploited to predict locations of cell-type-specific enhancers. Sensitivity to nuclease digestion is a standard method to identify regulatory elements or open chromatin regions, but previously this approach has been limited to small portions of the genome (7). However, recent advances have allowed the mapping of nuclease hypersensitivity genome-wide using microarrays or high-throughput sequencing (1). In fact, the accessible chromatin landscape of the human genome has recently been mapped in more than 100 different cell types by the ENCODE team (8).

Nuclease hypersensitive sites are flanked by nucleosomes, and like promoters, histone modification patterns can be used as indicators of enhancer functions. For instance, histone 3 lysine 4 monomethylation (H3K4me1) marks enhancers but includes both active and inactive or poised regulatory regions (9-12). Active enhancers are enriched for binding of the acetyltransferase p300 and histone 3 lysine 4 acetylation (H3K4Ac). In contrast, poised enhancers are flanked by H3K27me3 (9, 10). The predictive ability of the p300-based enhancer signature has been confirmed using a large series of transgenic mice (11). Another unexpected finding is evidence of transcription at functional enhancers (13). These transcripts at p300/CBP bound regions are designated enhancer RNA (eRNA). Measuring eRNAs has come to be used as a mark of enhancer activity (12). Considering the combinatorial nature of gene regulation, no single histone modification or nuclease hypersensitivity sites alone will likely provide the most complete annotation of enhancers (6). Integrating multiple histone modifications with coactivator p300, hypersensitive sites, and eRNA occupancy is probably the more comprehensive signature of active enhancers.

Although there have been tremendous advances in our ability to enumerate enhancers and other dynamic marks in junk DNA, linking these changes directly to the machinery involved in cellular differentiation has been more challenging. As we hope will be evident, hematopoietic cells and CD4⁺ T cells in particular represent a powerful system for studying how the chromatin landscape is modified and how signal transduction can link to a dynamic epigenome and thereby help define cellular identity. Here we summarize recent studies in

CD4⁺ T-cell specialization, which help to elucidate the roles of the extrinsic and intrinsic factors on the evolving epigenomes and transcriptomes of helper T subsets.

Naive CD4⁺ T cells as pluripotent precursors: helper T cells have many options

Among hematopoietic cells, CD4⁺ T cells exhibit some of the most varied effector options. As this population sets the overall tone of the adaptive immune response, their range of capabilities is perhaps not surprising. At the outset of activation, CD4⁺ T cells make these choices by sensing environmental cues. Th1 cells are critical for host defense against intracellular pathogens such as viruses and bacterial infection and exclusively secrete the cytokine interferon- γ (IFN γ) (14, 15). Th2 cell responses are important for controlling helminthic parasites and secrete interleukin-4 (IL-4), IL-5, and IL-13 (15), factors that are important for mucosal barrier function. Th17 cells regulate responses against extracellular bacteria and fungi and aptly secrete the cytokine IL-17 (16-20). They are also important drivers of autoimmune disease (21). More recently, a population of CD4⁺ T cells termed T-follicular helper cells (Tfh) have been recognized, which have specific effector function helping B cells. Unlike other subsets, the most reliable way to identify Tfh cells is based on their location. Tfh cells are found within B-cell follicles or germinal centers (GCs), the main site where antibody-forming cells are generated (22). While IL-21 has been referred to as the signature cytokine for Tfh cells, these cells can also produce cytokines made by other subsets including IFN- γ , IL-4, IL-17, and IL-10 (22-24). Conversely, production of IL-21 is not limited to Tfh cells; it is produced by other subsets as well (25, 26).

Additional effector populations have been described, including Th9 and Th22 cells, which produce IL-9 and IL-22, respectively (27, 28). Although there is clear evidence that CD4⁺ T cells can differentiate to these populations *in vitro*, the *in vivo* role of such cells is a topic of ongoing research. Th9 cells may play a critical role in diseases such as asthma (27); however, IL-9 and IL-22 is also produced by cells other than CD4⁺ T cells (29, 30). Thus, understanding the importance of Th9 and Th22 as CD4⁺ T-cell subsets requires additional investigation.

Another key option for CD4⁺ T cells is to take on a regulatory function and become so-called T-regulatory (Treg) cells. Their primary function is to suppress inflammation and effector T-cell responses (31-34). Unlike other effector populations, CD4⁺ T cells can choose a regulatory fate either in the thymus or after activation in the periphery. The former are denoted natural Tregs (nTregs) and the latter induced Tregs (iTregs) (32, 34). Whether these subsets can be distinguished based on cell surface markers has been the topic of some debate. Recently, neuropilin-1, a transmembrane protein with roles in T-cell priming (35), was found to be expressed on nTregs but not iTregs in mice, suggesting this molecule is a useful marker to distinguish these Treg subsets (36, 37). Both produce the anti-inflammatory cytokines IL-10 and transforming growth factor- β (TGF- β), or can suppress effector T-cell responses by consuming IL-2, limiting access to this important effector CD4⁺ T-cell growth factor (33). In addition to Treg cells, there are other types of immunosuppressive CD4⁺ T cells including Tr1 cells (38); whether these cells truly represent a distinct subset versus a temporary state is unclear.

Sometimes you do not have to make up your mind

Early work *in vitro* argued that polarized Th1 and Th2 cells were phenotypically fixed states; that is, 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 (14, 39, 40). One way this is accomplished is the downregulation of cytokine receptors required

to sense the environmental cues that drive an opposing fate (41). However, there are many lines of evidence that challenge a strict view of helper T cells as distinct lineages. For instance it is not infrequent for CD4⁺ T cells to co-express more than one ‘signature’ cytokine, particularly *in vivo*. Th17 cells, for example, readily become IFN- γ producers (42-46). In fact, the conversion of IL-17 producers to IFN- γ producers is an important aspect of immunopathogenesis disease models and likely autoimmune disease in humans (44).

Because Tfh cells do not produce a unique ‘signature’ cytokine and can also express cytokines produced by other fates, it should come as no surprise that Tfh cells have been argued to exhibit considerable flexibility. *In vitro* generated IL-21-producing Tfh-like cells can easily be re-differentiated to make IFN- γ , IL-4, or IL-17 (47). Conversely, early in their differentiation, Th1 cells exhibit features of Tfh cells (26). Later though, Tfh features are repressed and the ‘Th1’ aspects dominate. *In vivo*, during the peak response of a helminth infection, all of the IL-4-producing cells found in the lymph node can be viewed as Tfh cells, based on their location in B-cell follicles (23, 48, 49). Similarly, during bacterial infection, expression of IFN- γ is readily apparent in Tfh cells (23). For this reason, viewing Tfh cells as a fate similar to Th1 or Th2 cells has been questioned. One can envision a model in which the IFN- γ or IL-4-producing cells retained in the lymph node can be considered Tfh cells, while those that exit this milieu represent bona fide polarized Th1 or Th2 cells. Exposure of CD4⁺ T cells to signals provided by B cells likely transition the cells retained in the lymph node to a stable Tfh phenotype (22, 50, 51). The precise sequence of signals necessary for a cell to acquire both Tfh and Th1 or Th2 characteristics are still unclear, and thus the plasticity of Tfh cells is an area of active research.

Another dramatic example of flexible cytokine expression is the finding that committed Th2 cells can be reprogrammed to become IL-4⁺IFN- γ ⁺ double producing cells in the context of adoptive transfer and viral infection (52). These so-called ‘Th2+1’ cells exhibit aspects of both Th1 and Th2 programs and challenged the original model of the stable Th1/Th2 paradigm. Thus, all CD4⁺ T cells may in fact be quite flexible rather than fixed and stable fates; though they are among the most plastic, flexibility is not limited to Th17 and Tfh cells.

The flexibility or stability of Tregs is perhaps one of the most controversial and studied areas of helper T-cell plasticity. Aside from the interesting basic implications, transfer of Tregs has been proposed as a therapeutic approach for autoimmune diseases and cases where limiting inflammation may be beneficial. If Treg cells are highly unstable, this approach will have little likelihood of success (53). Several studies have reported that a fraction of Treg cells lose their stability, particularly during inflammatory environments where cues can potentially influence a Treg to convert to an effector cell (54-58). However, other groups have argued that these unstable cells are not *bona fide* Tregs or that conversion to effector fates is an experimental artifact (59, 60). Finally, whether these cells are thymically derived nTregs or peripherally derived iTregs may complicate our ability to assess the stability or flexibility of the Treg cell fate. While this area of research is still under investigation and unresolved, there are clear examples where cells with suppressive capacity can in the right circumstances take on a more effector cell fate.

In this context, CD4⁺ T-cell ‘identity crisis’ – how helper T cells choose an effector function while maintaining the ability to be flexible – is the major focus of much current research of CD4⁺ T-cell differentiation. Understanding the molecular basis of helper T-cell specification and plasticity is a critical question both in terms of the basic science and also clinical/translational implications. As we discuss in detail, there are multiple mechanisms to repress alternative fates when a given fate is adopted. If so, why is it that CD4⁺ T-cell options are

carefully regulated, yet provide for the possibility of flexible gene expression? An obvious possibility is that flexible helper T cells may be beneficial to the host for dealing with more than one pathogenic micro-organism: bacterial super-infections are common sequelae of viral infections and poly-microbial sepsis occurs in seriously ill patients. With age and declining T-cell repertoires, flexibility in gene expression may also be advantageous. T-cell plasticity may represent the tuning of the effector response as environmental cues change. Alternatively, the apparent flexibility might also represent specialization within the populations. This cellular heterogeneity could allow for adaptation to responses as environmental cues vary. This hypothesis would fit nicely into a dissection of responses in the peripheral lymph node organs versus the tissues, such as the gut or skin environment. Regardless of the reason, the many examples of T-cell plasticity suggest that there are multiple mechanisms that allow for this flexibility in effector function. As discussed in this review, these mechanisms include the regulation of the transcriptome through carefully controlled expression of transcription factors and by modulating the epigenomic landscape, both of which fundamentally influence the 'identity' of the CD4⁺ T cell.

Seeking identity in a complex world

Sensing the environment

There are a number of factors that seem to contribute to CD4⁺ T-cell specification. One well recognized factor is the 'strength' of T-cell receptor (TCR) signaling (61-63). Several studies have demonstrated that strong TCR signaling promotes Th1 differentiation, whereas weak TCR signaling favors the Th2 cell fate during *in vitro* T-cell skewing assays. In addition, strong T-cell receptor signaling is reported to induce Th17 differentiation (64). Strong TCR signals can also reportedly promote Tfh cell fate commitment (65). nTreg differentiation in the thymus is also thought to be the result of antigens that provide signals stronger than those that drive generation of conventional T cells but weaker than antigens that drive negative selection (66). Generation of iTregs on the other hand has been associated with weaker TCR signals (34, 67-70). How 'strong' signals can drive the differentiation of diverse subsets is difficult to understand, particularly *in vivo*, and the precise mechanistic underpinnings of these phenomena remain obscure.

In addition to TCR signaling, ambient environmental cues to which an activated T cell is exposed are key factors in helper cell specification. The secreted cytokines produced by both innate and adaptive immune cells are among the most important and relevant specifying factors. A large proportion of these cytokines, though not all, signal via receptors that belong to the Type I/II cytokine receptor superfamily (71). This family of receptors utilizes the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway signaling pathway to translate the extracellular cytokine signals into meaningful gene transcription programs, which promote differentiation of a given T-cell subset (72). Within the STAT family, there are seven DNA binding proteins (STAT1-5a, 5b, and 6) that function as transcription factors (73). The conventional view focuses on the distinct role of STATs as factors that drive T-cell differentiation in the presence of a given cytokine (72). However, the reality is more subtle and complex. Differentiating helper T cells are exposed to more than one cytokine during immune and inflammatory responses. In addition, most cytokines do not just activate a single STAT; most activate multiple STATs. Recently, the complex functions of STATs have become better appreciated; they do much more than just bind promoter elements and induce transcription. Indeed, the negative regulatory role of STATs in helper cell differentiation has become increasingly apparent.

Classically, we link IL-12-dependent activation of STAT4 with generation of Th1 cells, but STAT1 activated in response to IFN- γ is also a relevant player (74). Additional studies have also implicated IL-2 and STAT5 as contributors to Th1 cell differentiation (75). For Th2

cells, IL-4 and STAT6 promote differentiation, and the absence of STAT6 abrogates *in vitro* Th2 cell differentiation (74). However, Th2 cells are generated *in vivo* without IL-4 or STAT6 (76). Accordingly, STAT5 is also a contributor to Th2 differentiation. IL-2-dependent activation of STAT5a and STAT5b causes these factors to bind and transactivate the *Il4* and *Il4ra* genes thereby facilitating Th2 differentiation (77-81). IL-6 and STAT3 can also promote Th2 differentiation (82-84).

STAT3 is particularly important for the differentiation of Th17 cells, and without STAT3, Th17 differentiation is severely impaired (85-89). The failure to generate Th17 cells is an important feature of hyperimmunoglobulin E syndrome (HIES) or Job's syndrome, a disease that is due to dominant negative *STAT3* mutations (90, 91). IL-6 and IL-23 both activate STAT3, and along with IL-1 and TGF- β , these cytokines drive IL-17 production (92).

IL-6 and IL-21, again acting via STAT3, can promote the generation of Tfh-like cells (22, 93, 94). However, *in vivo* the absence of IL-6, IL-21, or STAT3 does not ablate Tfh cell development (93, 95-100). In this regard, it is notable that IL-12 and STAT4 promote Tfh cell formation both *in vitro* and *in vivo*, although sustained activation represses Tfh features (26, 100-102).

Finally, STAT5, activated by IL-2, is critical for Treg cell differentiation. In contrast to other STATs and their associated subsets, STAT5A/B are entirely non-redundant for Treg cells. In the absence of STAT5A/B, the differentiation of this subset is completely abrogated.

Although STATs were originally discovered as positive regulators of IFN-induced genes, their ability to inhibit gene expression is increasingly apparent. STAT4 can inhibit Th2 cell formation while promoting Th1 cell differentiation (74). Conversely, STAT6 inhibits Th1 cells while driving the Th2 cell fate (74). Although STAT5 promotes Treg, Th1, and Th2 cell differentiation, it is a negative regulator of Th17 and Tfh cells (103-106), again highlighting the dual role of STATs as both activators and inhibitors. In contrast, STAT3 activation inhibits iTreg differentiation (107, 108). Thus, for the Treg and Th17 fates, STAT5 and STAT3 have opposing roles. STAT1 is also important negative regulator of Th17 differentiation (109, 110). This is vividly demonstrated in the primary immunodeficiency disorder chronic mucocutaneous candidiasis. This disorder is characterized by recurrent fungal infections and impaired Th17 cell generation and is due to gain-of-function *STAT1* mutations (111). Thanks to new technologies, discussed in detail below, we can map precisely how STATs inhibit gene expression and antagonize the function of other STATs (112, 113). In summary, the complex nature of environmental cytokine cues to which a differentiating CD4⁺ T cell is exposed greatly influences the activation of different STAT family members, and they in turn are major contributors to the outcome of CD4⁺ T-cell fate determination.

Acquiring master regulators

In addition to the STAT family of transcription factors, each helper cell fate expresses a lineage-defining 'master regulator' transcription factor. Classically, these factors are defined as being both necessary and sufficient for characteristic features of the various subsets. In the conventional view, a single master regulator is expressed in a given helper T-cell subset (Fig. 1). Typically, T-bet (encoded by *Tbx21*) (114, 115), GATA-binding protein 3 (GATA3) (116), retinoic acid receptor-related orphan receptor- γ t (Ror γ t) (117), SFFV proviral integration 1 (PU.10) (118), B-cell lymphoma 6 (Bcl6) (119, 120), and forkhead box P3 (FoxP3) (121, 122) are referred to as the master regulators of Th1, Th2, Th17, Th9, Tfh, and Treg cells, respectively. However, recent emerging data have demonstrated that, like the signature cytokines, the expression of T-cell master regulators is more complicated

than originally appreciated. Indeed, the extent to which the nomenclature master regulator is apt is being revisited.

Unlike STAT family proteins, which are expressed in all subsets, master regulators are either not expressed in naive T cells or expressed at low levels. In many cases, their expression is driven by TCR signals and STATs. Under the influence of cytokines and other environmental cues, the dominant expression of one master transcription factor over the others can promote a specific T-cell fate, at least this was the classical model of T-cell differentiation. What complicates these initial views of master regulators is that their patterns of expression are substantially more complicated than the simple model equating a given subset with a single lineage-defining master regulator. Many examples of stable co-expression in fully differentiated cells now challenge this conventional model. For example, T-bet and GATA3 are transiently co-expressed in recently activated CD4⁺ T cells and can functionally interact, limiting the action of Gata3 (123). It is important though to distinguish this temporary and transitional type of co-expression from stable co-expression in a differentiated population of cells.

Treg cells are good example of the latter. Simultaneous expression of FoxP3 with T-bet, GATA3, or Bcl6 has been documented. This has been argued to be functionally relevant for control of Th1, Th2, and Tfh cell responses, respectively (57, 124-129). As STATs are important inducers of master regulators, it comes as no surprise that STAT1 is important for Treg cells to express T-bet via sensing the cytokines IL-12 or IL-27 (129, 130). Similarly, although STAT3 is not a master regulator, its expression in FoxP3⁺ Treg cells is important for suppression of Th17 responses (131). Together, these findings suggest that co-expression of master regulators may create populations of cells that are sub-specialized for specific functions of CD4⁺ T-cell subsets (Fig. 2A).

Somewhat counter-intuitively, T-bet and GATA3 can be stably co-expressed in previously committed Th2 cells to create a population termed Th2+1 that has features of both Th1 and Th2 cells (52). This example of co-expression suggests a way for CD4⁺ T cells to ensure some flexibility that may be crucial to host defense against diverse microbial pathogens, rather than a sub-specialized population that has unique functional properties (Fig. 2B).

T-bet and Bcl6 can also be simultaneously expressed (26, 132-134). These studies suggested that low levels of T-bet can be co-expressed with low levels of Bcl6 in CD4⁺ T cells. However, at higher concentrations these factors are mutually antagonistic. Thus, the balance between T-bet and Bcl-6 expression could be important for the decision between a Th1 and Tfh cell (Fig. 2C).

These examples of co-expression make it appropriate to re-visit and re-define our views of master regulator. Functionally, helper cells have the ability to co-express more than one master regulator to provide something necessary for the immune response. But the molecular mechanisms of how this co-expression can exist in certain settings, and how it shapes the identity of the cell both from a transcriptomic and epigenomic view likely underlies the basis for helper T-cell plasticity.

Towards a transcription factor network for helper T cells

While STATs and master regulators are critical for helper cell differentiation, it is overly simplistic to try to explain the diverse functionalities of helper T cells based on these two classes of factors. In reality, specification requires a cohort of critical transcription factors working in concert. During T-cell development, CD4⁺ T cells express an array of transcription factors that dynamically change over the course of commitment in the thymus, allowing them to diverge from CD8⁺ T cells (135). Factors such as Thpok, Runx3, Runx1,

Ets1, Tox, and the E proteins E2A and HEB are all induced at discrete steps to drive commitment to either the CD4⁺ or CD8⁺ lineage. Importantly, GATA3, the so-called master regulator of Th2 cells, has critical roles in thymic development as well (136). Thus, any given transcription factor may have stage-specific functions.

In addition to the factors required for development, several other transcription factors have been described that are critical for CD4⁺ T cells but are not specifically required for only one subset. Interferon regulatory factor 4 (IRF4) is important for the differentiation of Th2, Treg, Th17, Th9, and Tfh cells (137-141). To function, IRF4 complexes with members of the AP1 family, making family members like BATF and c-Maf necessary for several CD4⁺ T-cell subsets (142-146). Additional transcription factors that are important for Th1 cells include Hlx, Runx3, and the Ets family members (147-149). In addition to c-Maf, the AP1 family member JunB and the transcription factor Gfi-1 are required for Th2 cells (150, 151). Recently, HIF1, Runx1, Aiolos, and Fosl2 have all been demonstrated to be important for Th17 cells (144, 152-154).

The recognition that helper T cells express many key transcription factors has been aided by improved technologies for measuring their simultaneous expression. This allows us to view helper T cells with a 'wide-angle lens' rather than taking narrowly focused snapshots. The complexity of the transcription factor networks is daunting to consider; however, at this point it is rather naive to simply measure one transcription factor, master regulator or otherwise, and try to make strong functional conclusions. A much more reasonable view is to simply accept the intricacy of transcription factor action, knowing that these factors work in multimolecular complexes. Thus, we need to appreciate that the master regulators and STATs exert their effect in the context of other transcription factors needed for CD4⁺ T-cell identity and try to be comprehensive in assessing the expression of all these factors.

CD4⁺ T-cell transcriptomic and epigenetic considerations

So far, we outlined a number of transcription factors that play pivotal roles in establishing the identity of helper T cells. But what do these transcription factors really do towards helper T-cell specification? The answer lies in their concerted impacts on the transcriptome and epigenome of T-helper subsets.

Global transcriptional profiling in T cells

Although CD4⁺ T-cell subsets are typically defined by their expression of cytokines and master regulator transcription factors, the question arises how many other genes are unique to each helper T-cell subset? For more than a decade, microarray based technologies have been the technology of choice for high-throughput transcriptome profiling in CD4⁺ T cells (155). These studies have indicated that hundreds of genes are differentially expressed among various T-helper subsets, many of which can be directly related to their differentiation and function (112, 155, 156). Despite their routine use, expression microarrays have limitations. These include hybridization and cross-hybridization artifacts and design constraints that prevent the detection of RNA splicing and previously unknown transcripts. In contrast, RNA sequencing (RNA-seq) provides detailed information about microRNAs (miRNAs), long non-coding RNAs (lncRNAs), unannotated genes, and splicing isoforms (157). This technology now provides a more complete catalogue of transcription and sequence based profiling of the transcriptome is slowly replacing expression microarrays.

Using RNA-seq technology, the transcriptomes during thymic T-cell development have been obtained (135). Unexpectedly, the largest differential gene expression occurred between double negative 2a (DN2a) and DN2b cells, not between DN1 and DN2, where Notch

signaling induces the first signs of T-lineage entry. The transcriptional profiling in Th17 cells also identified more than hundred genes to be either expressed or repressed in these cells compared to the naive state (144). Such RNA-seq datasets not only quantify gene expression levels but also provide detailed information about the choice of exons that is in use in different T-helper subsets (135).

RNA sequencing has allowed the repertoire of non-coding RNAs to be glimpsed for the first time. Among these categories of transcripts, miRNAs are small single-stranded non-coding transcripts that post-transcriptionally regulate their target genes. Drosha and Dicer are two major components that allow for miRNA processing and function (158). Loss of these factors affects antibody diversity in B cells and also leads to instability of helper T cells and consequently autoimmunity (158-160). A comprehensive profiling of miRNAs during lymphopoiesis revealed the underlying transcriptional forces that shape miRNA homeostasis (161). Various miRNAs were reported to play key roles in T-cell specification. For example, miR-125b contributes to enforcing a naive state of Th cells (162), whereas miR-182 promotes clonal expansion (163). By contrast, miR-10a attenuates the phenotypic conversion of iTregs into Tfh cells by targeting Bcl6 and the co-repressor Ncor2 (164).

The importance of a limited number of lncRNAs has long been appreciated (165). Examples include Xist and Air, which are associated with heterochromatin formation and imprinting (166, 167). Overall though, relatively few lncRNAs have been functionally characterized and even fewer have been studied in T cells. One lncRNA, Tmevpg1, located in relative proximity to the *Ifng* gene, positively controls expression of this key gene (168). Interestingly, STAT4 and T-bet are both regulators of Tmevpg1 expression. ENCODE has identified ten-thousand new lncRNAs (169). Thus, this area of T-cell biology is clearly in its infancy. Undoubtedly, there will be many more non-coding RNAs that will have an impact on helper cell specification, and we are only beginning to understand their roles in helper T-cell function.

Chromatin status of CD4⁺ T cells

As explained in the introduction, the expression of a given transcription factor may not be sufficient for it to exert its effect; its targets need to be accessible. In this way, the chromatin landscape permits transcription factors to act on the genome and thus both factors contribute to selective gene expression and acquisition of cell identity. In fact, it has long been recognized that CD4⁺ T cells respond to changes in their environments by reshaping the chromatin architecture of signature cytokine loci and other key genes expressed by effector CD4⁺ T cells (170-175).

The impact of histone modifications, nucleosome positioning, or DNA methylation in CD4⁺ T-cell differentiation has been established through the use of genetic deletions or knockdown approaches. Brahma-related gene 1 (BRG1)-containing complexes, which displace nucleosomes, are important for remodeling the *Ifng* locus and production of this cytokine is important for Th1 differentiation (176). The cohesin protein complex is also important for the maintenance of gene architecture (177). Knocking down just one component of this complex, RAD21, also causes a reduction of *IFNG* transcript levels (178). Conversely, the Trithorax complex functions as a histone 3 lysine 4 methyltransferase and thereby serves to repress gene expression. Impaired expression of the components of this complex are associated with aberrant Th2 differentiation (179, 180). Similarly, H3K9me3 is another important marker of gene silencing. Deletion of the H3K9 methylase results in inappropriate expression of Th1 genes in Th2 cells (181). Another key factor in silencing gene expression is DNA methylation. Conditional ablation of the DNA methyltransferase, DNMT1, or methyl-CpG binding domain protein 2 results in the inability of Th1 or Th2

cells to properly silence the expression of genes typically expressed in the opposing lineage (91-93).

Not surprisingly, epigenetic modifications extend beyond the control of the cytokine genes, affecting the expression of the key transcription factors associated with helper T-cell differentiation. For example, a conserved CpG rich region resides upstream of the FoxP3 promoter (182). This region is partly methylated in iTreg cells but completely demethylated in nTreg cells. This helps explain the relative stability of expression of Foxp3 in nTreg cells (183-185).

Recent developments in deep sequencing technologies have now replaced the painstaking mapping of chromatin modifications at individual loci with genome-wide catalogues. In particular, chromatin immunoprecipitation followed by sequencing (ChIP-seq) is widely used for profiling histone tail modifications, distribution of chromatin-associated proteins, and binding of transcription factors (186). Genome-wide mapping of the trimethylation status of naive CD4⁺ T cells, and fully polarized Th1, Th2, Th17, iTreg, and nTreg cells has now been accomplished (56). Consistent with the standard ‘lineage commitment’ view of helper cell differentiation, signature cytokine genes exhibit unopposed permissive marks (H3K4me3) in the appropriate subsets (e.g. *Ifng* in Th1 cells) and repressive marks (H3K27me3) in other subsets that do not express these cytokines. Genes other than cytokine genes also exhibited this pattern of regulation. Surprisingly though, genes encoding master regulators such as *Tbx21*, *Gata3*, *Bcl6*, *Runx3*, and *Prdm1* have more complex marks. In many cases these genes exhibit “bivalent poised domains”, meaning that both accessible and repressive marks are present (56). This helps explain why T-bet can be expressed in Treg, Th17, and even Th2 cells (30, 38, 100). It also explains the flexible expression of Bcl6 and how a novel GATA3- and ROR γ t-expressing subset of Th2 memory/effector cells can arise (187). Thus, interrogating the epigenetic modifications of genes encoding cytokines and transcription factors provide insights into stability and flexibility of expression.

How transcription factors act on the chromatin environment and transcription of genes

Knowing that both the transcriptomes and chromatin landscapes of differentiating T cells are highly dynamic, an obvious next question is how different transcription factors impact on these aspects of T-cell biology. To what extent do STATs and master regulator factors modify chromatin changes and transcription and are their effects direct or indirect?

Action of the environmental sensors

As critical sensors of the cytokine milieu that shape T-cell differentiation, it might be anticipated that various STATs can have a substantial effect on transcriptomic and epigenomic changes. However, it was an open question as to which factor would have a more pronounced effect: STATs or master regulators. Would these factors influence both transcription and epigenetic modifications? The role of STATs on transcriptomic changes has been documented using microarray technology and the major impact of STATs is very clear (65, 102). However, microarray technology does not allow one to distinguish direct versus indirect effects. In addition to measuring genome wide changes in histone modifications, ChIP-seq can be used to measure genome wide transcription factor binding. Coupling this information with transcriptional profiling by microarrays or RNAseq and histone ChIP-seq in wildtype versus transcription factor-deficient cells, one can begin to achieve a more complete view of transcription factor action.

Genome-wide binding studies for STAT3, STAT4, STAT5, and STAT6 proteins identified target genes of these transcription factors in Th1, Th2, and Th17 cells (52, 65, 102, 103).

While most of STAT4 binding sites (80%) occurred outside the promoter regions in Th1 cells, 4000 genes were directly bound by STAT4. Similar results were found for STAT6 in Th2 cells (112). Analysis of global STAT3 binding sites in Th17 cells also revealed thousands of genes that are bound by this protein (144, 188). Thus, the expectation would be that STATs have potential for having broad effects on the genome; this turns out to be the case.

STAT4 activates genes important for Th1 function such as *Tbx21*, *Ifng*, and *Il12rb2* (189, 190). Likewise, STAT6 drives *Gata3*, *Il4*, *Il4ra*, and other genes necessary for Th2 cells (191). STAT5 plays a pivotal role in regulating Treg differentiation by directly binding the *Foxp3* gene and inducing its expression (192). STAT3 positively regulates many genes important for Th17 cell formation and function, including *Rorc*, *Il17a/f*, and *Il23r* (188, 193). In addition, STAT3 is a strong activator of *Il21*, expressed by both Th17 and Tfh cells, suggesting why STAT3 has a role in both of these subsets. It should be emphasized that of the STAT-bound genes, only a subset of genes are highly dependent upon the cognate STAT. Overall, less than 25% of STAT-bound genes require STAT for gene regulation (112, 144). This makes sense however, when one considers that STATs are just one of many families of transcription factors in the genome.

In addition to their action in promoting gene expression, STAT deficiency has elucidated their role in inhibiting gene expression (194). Prior to the generation of ChIP-seq datasets however, it was not possible to discern direct versus indirect effects. A surprise from these new data was the extent to which STATs have a direct repressive function. STAT4 and STAT6 can both bind to target genes and reciprocally antagonize each other's function in Th1 and Th2 cells. For example, *Ccr8* is preferentially expressed in Th2 cells in a STAT6-dependent manner, whereas STAT4 inhibits this gene in Th1 cells (112). In Th17 cells, IL-2-activated STAT5 directly competes with IL-6-activated STAT3, binding the same genomic elements and thereby limiting gene expression (113). STAT5 can also bind and repress the *Bcl6* locus, using the same manner of competition with STAT3 (134). STAT3 also functions to limit FoxP3 expression (86, 103, 107, 108, 195, 196); however in this case, the direct versus indirect actions of STAT3 have not been comprehensively defined. An example of the indirect action of STAT3 is its role in limiting IL-2 by induction of the Ikaros family protein Aiolo, which acts directly to represses the *Il2* gene (154). Thus, STATs have multiple ways of mediating repression of T-cell subsets, either by directly competing with activators or by inducing expression of other transcriptional repressors.

Although STAT binding occurs at promoters, they also bind within genes, in 3' untranslated regions and at distal sites. This suggested the possibility that in addition to just regulating transcription, STATs might also influence the epigenetic landscape in which genes reside. In fact, this is the case. STATs have major effects on both accessible and repressive marks. Globally, STAT4 has a significant effect on H3K4me3. In contrast, the major genome wide action of STAT6 had a greater effect on the repressive histone mark H3K27me3. In some cases, STAT6 deficiency promoted the appearance of repressive marks for genes expressed in Th2 cells (e.g. *Il4*, *Gata3*, and *Il4ra*), thus a major action of STAT6 is to remove repressive marks (112).

We now know that STATs can impact target genes in three ways: by affecting transcription and epigenetic modifications (~5%), by only affecting transcription (~11%), and by only affecting epigenetic modifications (~20%). For most STAT-bound genes however, there is no impact on either transcription or epigenetics (~64%). These observations suggest that STATs act on genes via multiple mechanisms, presumably in concert with other transcription factors (174).

Action of master regulators

The fact that the master regulators were both necessary and sufficient for differentiation of each corresponding T-cell subset suggested that the action of these transcription factors might encompass many aspects of regulating helper cell identity. In fact, T-bet regulates thousands of genes in Th1 cells (26, 197, 198). Similar to STAT4, T-bet binds to the *Ifng* locus (199) and promotes its expression, as well as the loci for *Il12rb2* and *Cxcr3* (115, 148, 200). Global profiling of T-bet binding and its impact on transcription and epigenetics has now been accomplished (201). Roughly 6% of genes bound by T-bet are transcriptionally regulated by this factor, but overall the number of genes positively or negatively regulated by T-bet are comparable. For genes expressed in Th1 cells, T-bet binding correlates with H3K4me1. For genes normally expressed in Th2 cells, T-bet binding is associated with H3K27me3 (201). Unlike T-bet, GATA3 is constantly available at almost all stages of T-cell development and in differentiated T cells. However, the distribution of GATA3 occupancy varies greatly in different stages of T-cell development (135). In Th2 cells, GATA3 is required for the expression of many Th2 associated genes, such as *Il4*, *Il5*, *Il13*, and *Batf* (202). Globally, 60% of the genes that require GATA3 for transcription also exhibit GATA3 binding, arguing for direct mode action in a relatively large proportion of genes (202). However, many genes were found to be negatively regulated: 30% of the genes repressed by GATA3 were bound by this factor in Th2 cells. These data suggest that like the STATs and T-bet, GATA3 can act as both a transcriptional activator and repressor. Interestingly, GATA3 regulates both H3K4me2 and H3K27me3 at many target genes. In the absence of GATA3, *Il10* had more repressive marks, but at the *Tbx21* and *Ifng* loci, reduced H3K27me3 marks were observed. The global effect of GATA3 deletion on H3K4me2 and H3K27me3 has also been shown (202). Thus, T-bet and GATA3 effect gene expression via changes in transcription and epigenetic modification.

Ideally, one would like to compare the global epigenomic impact of T-bet and GATA3 with STAT4 and STAT6 (112, 201, 202). It is clear that deletion of these factors has an impact on the epigenome. However, usage of different epigenetic marks as well different computational approaches makes a direct comparison difficult. No doubt this information will be available in the not too distant future.

Unlike T-bet and GATA3, Ror γ t and Foxp3 have little effect on the epigenetic landscapes of their respective subsets (144, 203). Instead, their binding is highly enriched at promoters where they behave predominantly as direct activators or repressors. The designation as master regulator might imply broad acts on numerous genes. However, the new data argue that Ror γ t has a relatively focused mode of action serving as modulator rather than a master transcription factor. In fact, Ror γ t binding is associated with modest changes in gene expression in Th17 cells relative to Th0 cells (144). It positively regulates the expression of 68 genes but only strongly regulates a small number of core genes associated with the Th17 fate (e.g. *Il17a*, *Il17f*, and *Il23r*) (144). In addition, Ror γ t acts as a repressor, limiting expression of genes such as *Il10*, *Hif1a*, *Foxo1*, and *Il7r*, as well as genes associated with alternate fates, such as *Il4ra*, and *Il12rb2* (144). Thus, in contrast to other factors including STAT3, IRF4, or BATF, Ror γ t's direct functions are rather limited.

The absence of FoxP3 in Treg cells diminishes the expression of many Treg signature genes (204). However, gene expression is not abrogated, suggesting that additional factors contribute to regulation of expression. In fact, many partners of Foxp3 protein have been identified that may have a role in regulating Treg-specific gene expression (205). Like other factors, Foxp3 can either promote or repress gene expression both in Tregs and non-Tregs (192, 203, 206). Overall, similar numbers of genes are regulated by FoxP3, T-bet, or GATA3 (~200 genes) (192, 201-203). Although binding of FoxP3 is correlated with a variety of active and repressive histone marks, a major surprise is that deletion of Foxp3

does not have substantial impact on global chromatin status (203, 207). Rather, it appears that Foxp3 target genes are ‘prepared’ for Foxp3 arrival by other transcriptional factors, which act as ‘place holders’ (203, 207). The focused effect of FoxP3 is in line with its global enrichment at promoters (203).

Bcl6 is a transcriptional repressor that acts by binding to co-repressors and recruiting histone deacetylase complexes (208). Several co-repressors for Bcl6 have been identified, but the combinatorial effects of each of these co-repressors and the genes regulated by these complexes have not been elucidated (208). A handful of specific target genes have been identified in T cells including *Prdm1* (which encodes the transcriptional repressor Blimp1), *Tbx21*, *Gata3*, *Rorc*, and *Il5* (22, 209-211). Unfortunately, Chip-seq of Bcl6 in T cells has not been reported, and thus the genome-wide binding targets and effects of Bcl6 are unknown.

While it is clear that the master regulators are important for T-cell subset specific gene expression, it is apparent that they do not have pervasive effects on gene expression and chromatin landscapes; on the contrary, their effects are rather focused. In fact, current data suggest the master regulators control the transcription of a select group of gene modules associated with each subset while repressing a roughly similar number of genes important for other fates. Yet, it appears that the cell may already be ‘primed’ by other factors such as the STATs or additional transcription factors that mediate appropriate epigenetic changes, so that the master regulators can take advantage of this newly accessible landscape.

Enumerating enhancers and defining function of Junk DNA in T cells

While alternative lineage decisions involve the activation of different genes, a major determinant of tissue-specific gene expression and hence cell identity is the existence of enhancer elements. The *Ifng* and *Il4* loci are good examples of the complicated architecture of finely regulated lineage-specific genes (172, 212). For instance, a conserved noncoding sequence in the distal site of the *Il4* locus is critical for IL-4 expression in Tfh cells but not in Th2 cells (213). In addition, intrachromosomal interactions of enhancers with promoters in the Th2 cytokine locus are critical for optimal expression of these cytokines (214). Likewise, a *Foxp3* enhancer (CNS1) is essential for peripheral Tregs but dispensable for natural Treg cell generation; notably, this element is only active only placental mammals (215).

Until recently, obtaining global views of enhancers has not been possible. However, deep sequencing approaches using DNase hypersensitivity and histone modifications now permit such views. This technology has been successfully employed to interrogate T cells and CD4 subsets (135, 144, 203, 216). Global mapping of p300 binding, which marks active enhancers, indicates that there are thousands of functional but unique *cis*-regulatory elements in Th1, Th2, and Th17 cells (144, 216). By contrast, mapping of open chromatin regions by genome-wide DNaseI hypersensitive sites argues that the majority of accessible regulatory regions in Tregs are already open in naïve CD4 T cells. Whether nucleosomes flanking the open chromatin sites have distinct patterns of histone modifications in Tregs and naïve T cells needs to be investigated.

It might be anticipated that master regulators would have profound positive effects on lineage-specific enhancers, but this has not turned out to be the case. For example, T-bet is largely dispensable for active Th1-specific enhancers. However, it has a negative impact on enhancers of opposing fates, suggesting a predominant role as a repressor (216). Similarly, Ror γ t has a surprisingly small regulatory footprint: the absence of Ror γ t did not change the p300 occupancy in Th17 cells (144). In addition, Rudensky and colleagues (203) uncovered that the enhancer landscape of Tregs is independent of Foxp3. Rather, Foxp3 defines Treg

cell functionality in an opportunistic manner by exploiting the available enhancer landscape instead of establishing a new one (203). In other words, Foxp3, like T-bet and Ror γ t, plays a limited role in establishing the enhancer landscape of Tregs. These three studies support the notion that the so-called master regulators have focused roles particularly on the promoter of their target genes and are not capable of globally organizing the chromatin signature of enhancers.

While the functional importance of enhancers is well appreciated, what has been less clear is the factors that drive the activation of these regulatory elements. STAT3, STAT4, and STAT6 proteins are shown to be the major organizers of the enhancer landscapes in Th17, Th1, and Th2 cells, respectively (144, 216). Importantly, reconstitution of STAT4- and STAT6-deficient cells with T-bet and GATA3 failed to recover the active enhancer landscapes (216). In addition, transcriptomic changes mediated by STATs correlated well with STAT-dependent changes in p300 binding. STATs can also promote the loss of p300 recruitment at enhancers of opposite fates, indicative of the roles of STATs in both limiting and promoting gene expression. Together, these studies suggest that STATs act as mediators of crosstalk between enhancers and the cellular environment.

It has been reported that the poised enhancer landscape is likely premarked in multipotent progenitors (217). In line with this report, deletion of STATs did not lead to a huge loss in H3K4me1-positive poised enhancer landscape (216). These results suggest that ‘pioneering factors’ other than STATs are likely responsible for establishing the poised elements. In fact, IRF4 and BATF, an AP-1 family protein, act as pioneering factors in Th17 cells (144). This study demonstrated that these two proteins cooperatively bind to Th17 regulatory elements even in non-polarized T cells receiving TCR stimulation alone. In addition, in the absence of IRF4 or BATF, Th17-specific regulatory elements were shown to be less accessible (144). Pre-patterning of chromatin by AP-1 family proteins has been reported in other cell types (218). The cooperation of IRF4 and BATF in Th17 cells has also been demonstrated in another study (143). Overall, these studies suggest a stepwise process of the enhancer firing in which the establishment of poised enhancers by pioneering factors precedes the action of sensors of the environment such as STATs. In this scheme, master regulators act as modulators of expression and have focused impacts on promoters (Fig. 3).

Concluding remarks

Transcription factors like T-bet and Ror γ t were dubbed master regulators when our ability to measure genomic and epigenomic changes was limited. Exploration of the genes encoding master regulators revealed their bivalent chromatin status in all lineages, suggesting they are poised for expression only under the right cytokine setting. Our ability to catalogue functional elements of the non-coding part of the genome has provided a new and very different perspective of the relative roles of various factors in T cell specialization. A major surprise has been the limited functions of the master regulators. Recent genome-wide studies argue for focused rather than pervasive functions of these factors. This perspective fits better with the evolving views of T-cell plasticity and T-cell acquisition of specialized functions. It is also clear that the T-helper cell epigenome can be altered by changes in the cellular environment, such as TCR stimulation or the cytokine milieu, sensed by pioneering factors or STAT proteins. Factors other than master regulators set the epigenetic stage, and master regulators exploit the heavy lifting of those other factors. There may be considerable logic in this division of labor. If master regulators had global impacts on chromatin landscapes, the T-cell program might be ‘stuck’, with little opportunity to respond to changes in the environment. Instead, current data argue that the epigenetic landscape of master regulator genes allows flexibility, and the action of these factors is discrete rather than pervasive. Whether this scenario more accurately reflects the regulation of helper cell

specifications remains to be determined, but there is no doubt that this exciting and important area will continue to be the focus of many future studies. The lessons learned will undoubtedly be of interest in terms of the basic mechanisms that they reveal, and will hopefully provide new insights on how we might reprogram immune responses to optimize vaccine and treat immune-mediated disease.

Acknowledgments

This work was supported by NIH/NIAMS Intramural Research Program (IRP), the JSPS Research Fellowship for Japanese Biomedical and Behavioral Researchers at NIH (K.H.) and PRAT (A.P.).

References

1. Bulger M, Groudine M. Functional and mechanistic diversity of distal transcription enhancers. *Cell*. 2011; 144:327–339. [PubMed: 21295696]
2. Djebali S, et al. Landscape of transcription in human cells. *Nature*. 2012; 489:101–108. [PubMed: 22955620]
3. Natoli G. Maintaining cell identity through global control of genomic organization. *Immunity*. 2010; 33:12–24. [PubMed: 20643336]
4. Maston GA, Evans SK, Green MR. Transcriptional regulatory elements in the human genome. *Annu Rev Genomics Hum Genet*. 2006; 7:29–59. [PubMed: 16719718]
5. Prabhakar S, Noonan JP, Paabo S, Rubin EM. Accelerated evolution of conserved noncoding sequences in humans. *Science*. 2006; 314:786. [PubMed: 17082449]
6. Buecker C, Wysocka J. Enhancers as information integration hubs in development: Lessons from genomics. *Trends in Genetics*. 2012; 28:276–284. [PubMed: 22487374]
7. Gross DS, Garrard WT. Nuclease hypersensitive sites in chromatin. *Annu Rev Biochem*. 1988; 57:159–197. [PubMed: 3052270]
8. Thurman RE, et al. The accessible chromatin landscape of the human genome. *Nature*. 2012; 489:75–82. [PubMed: 22955617]
9. Creighton MP, et al. Histone H3K27ac separates active from poised enhancers and predicts developmental state. *Proc Natl Acad Sci USA*. 2010; 107:21931–21936. [PubMed: 21106759]
10. Rada-Iglesias A, Bajpai R, Swigut T, Bruggmann SA, Flynn RA, Wysocka J. A unique chromatin signature uncovers early developmental enhancers in humans. *Nature*. 2011; 470:279–283. [PubMed: 21160473]
11. Visel A, et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature*. 2009; 457:854–858. [PubMed: 19212405]
12. Wang D, et al. Reprogramming transcription by distinct classes of enhancers functionally defined by eRNA. *Nature*. 2011; 474:390–397. [PubMed: 21572438]
13. Kim TK, et al. Widespread transcription at neuronal activity-regulated enhancers. *Nature*. 2010; 465:182–187. [PubMed: 20393465]
14. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol*. 1989; 7:145–73. [PubMed: 2523712]
15. Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. *Nature*. 1996; 383:787–793. [PubMed: 8893001]
16. Stockinger B, Veldhoen M, Martin B. Th17 T cells: linking innate and adaptive immunity. *Semin Immunol*. 2007; 19:353–361. [PubMed: 18023589]
17. Harrington LE, et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol*. 2005; 6:1123–1132. [PubMed: 16200070]
18. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity*. 2006; 24:179–189. [PubMed: 16473830]
19. Bettelli E, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature*. 2006; 441:235–238. [PubMed: 16648838]

20. Mangan PR, et al. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature*. 2006; 441:231–234. [PubMed: 16648837]
21. Miossec P, Korn T, Kuchroo VK. Interleukin-17 and type 17 helper T cells. *The N Engl J Med*. 2009; 361:888–898.
22. Crotty S. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol*. 2011; 29:621–663. [PubMed: 21314428]
23. Reinhardt RL, Liang HE, Locksley RM. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat Immunol*. 2009; 10:385–393. [PubMed: 19252490]
24. Hsu HC, et al. Interleukin 17-producing T helper cells and interleukin 17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice. *Nat Immunol*. 2008; 9:166–175. [PubMed: 18157131]
25. Wei L, Laurence A, Elias KM, O'Shea JJ. IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner. *J Biol Chem*. 2007; 282:34605–34610. [PubMed: 17884812]
26. Nakayama S, et al. Early Th1 cell differentiation is marked by a Tfh cell-like transition. *Immunity*. 2011; 35:919–931. [PubMed: 22195747]
27. Stassen M, Schmitt E, Bopp T. From interleukin-9 to T helper 9 cells. *AAAnn NY Acad Sci*. 2012; 1247:56–68.
28. Duhon T, Geiger R, Jarrossay D, Lanzavecchia A, Sallusto F. Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. *Nat Immunol*. 2009; 10:857–863. [PubMed: 19578369]
29. Wilhelm C, Turner JE, Van Snick J, Stockinger B. The many lives of IL-9: a question of survival? *Nat Immunol*. 2012; 13:637–641. [PubMed: 22713829]
30. Zenewicz LA, Flavell RA. Recent advances in IL-22 biology. *Int Immunol*. 2011; 23:159–163. [PubMed: 21393631]
31. Shevach EM. CD4+ CD25+ suppressor T cells: more questions than answers. *Nat Rev Immunol*. 2002; 2:389–400. [PubMed: 12093005]
32. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell*. 2008; 133:775–787. [PubMed: 18510923]
33. Josefowicz SZ, Lu LF, Rudensky AY. Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol*. 2012; 30:531–564. [PubMed: 22224781]
34. Josefowicz SZ, Rudensky A. Control of regulatory T cell lineage commitment and maintenance. *Immunity*. 2009; 30:616–625. [PubMed: 19464984]
35. Tordjman R, et al. A neuronal receptor, neuropilin-1, is essential for the initiation of the primary immune response. *Nat Immunol*. 2002; 3:477–482. [PubMed: 11953749]
36. Yadav M, et al. Neuropilin-1 distinguishes natural and inducible regulatory T cells among regulatory T cell subsets in vivo. *J Exp Med*. 2012; 209:1713–1722. [PubMed: 22966003]
37. Weiss JM, et al. Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3+ T reg cells. *J Exp Med*. 2012; 209:1723–1742. [PubMed: 22966001]
38. Pot C, Apetoh L, Kuchroo VK. Type 1 regulatory T cells (Tr1) in autoimmunity. *Semin Immunol*. 2011; 23:202–208. [PubMed: 21840222]
39. O'Shea JJ, Paul WE. Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. *Science*. 2010; 327:1098–102. [PubMed: 20185720]
40. Yamane H, Paul WE. Memory CD4+ T cells: fate determination, positive feedback and plasticity. *Cell Mol Life Sci*. 2012; 69:1577–1583. [PubMed: 22481436]
41. Zhu J, Paul WE. CD4 T cells: fates, functions, and faults. *Blood*. 2008; 112:1557–1569. [PubMed: 18725574]
42. Shi G, Cox CA, Vistica BP, Tan C, Wawrousek EF, Gery I. Phenotype switching by inflammation-inducing polarized Th17 cells, but not by Th1 cells. *J Immunol*. 2008; 181:7205–7213. [PubMed: 18981142]
43. Muranski P, et al. Tumor-specific Th17-polarized cells eradicate large established melanoma. *Blood*. 2008; 112:362–373. [PubMed: 18354038]

44. Bending D, et al. Highly purified Th17 cells from BDC2.5NOD mice convert into Th1-like cells in NOD/SCID recipient mice. *J Clin Invest*. 2009; 119:565–572. [PubMed: 19188681]
45. Lee YK, et al. Late developmental plasticity in the T helper 17 lineage. *Immunity*. 2009; 30:92–107. [PubMed: 19119024]
46. Hirota K, et al. Fate mapping of IL-17-producing T cells in inflammatory responses. *Nat Immunol*. 2011; 12:255–263. [PubMed: 21278737]
47. Lu KT, et al. Functional and epigenetic studies reveal multistep differentiation and plasticity of in vitro-generated and in vivo-derived follicular T helper cells. *Immunity*. 2011; 35:622–632. [PubMed: 22018472]
48. King IL, Mohrs M. IL-4-producing CD4⁺ T cells in reactive lymph nodes during helminth infection are T follicular helper cells. *J Exp Med*. 2009; 206:1001–1007. [PubMed: 19380638]
49. Zaretsky AG, Taylor JJ, King IL, Marshall FA, Mohrs M, Pearce EJ. T follicular helper cells differentiate from Th2 cells in response to helminth antigens. *J Exp Med*. 2009; 206:991–999. [PubMed: 19380637]
50. Deenick EK, et al. Follicular helper T cell differentiation requires continuous antigen presentation that is independent of unique B cell signaling. *Immunity*. 2010; 33:241–253. [PubMed: 20691615]
51. Goenka R, et al. Cutting edge: dendritic cell-restricted antigen presentation initiates the follicular helper T cell program but cannot complete ultimate effector differentiation. *J Immunol*. 2011; 187:1091–1095. [PubMed: 21715693]
52. Hegazy AN, et al. Interferons direct Th2 cell reprogramming to generate a stable GATA-3⁺T-bet⁺ cell subset with combined Th2 and Th1 cell functions. *Immunity*. 2010; 32:116–128. [PubMed: 20079668]
53. Bailey-Bucktrout SL, Bluestone JA. Regulatory T cells: stability revisited. *Trends Immunol*. 2011; 32:301–306. [PubMed: 21620768]
54. Komatsu N, Mariotti-Ferrandiz ME, Wang Y, Malissen B, Waldmann H, Hori S. Heterogeneity of natural Foxp3⁺ T cells: a committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity. *Proc Natl Acad Sci USA*. 2009; 106:1903–1908. [PubMed: 19174509]
55. Tsuji M, et al. Preferential generation of follicular B helper T cells from Foxp3⁺ T cells in gut Peyer's patches. *Science*. 2009; 323:1488–1492. [PubMed: 19286559]
56. Wei G, et al. Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4⁺ T cells. *Immunity*. 2009; 30:155–167. [PubMed: 19144320]
57. Oldenhove G, et al. Decrease of Foxp3⁺ Treg cell number and acquisition of effector cell phenotype during lethal infection. *Immunity*. 2009; 31:772–786. [PubMed: 19896394]
58. Feng T, Cao AT, Weaver CT, Elson CO, Cong Y. Interleukin-12 converts Foxp3⁺ regulatory T cells to interferon-gamma-producing Foxp3⁺ T cells that inhibit colitis. *Gastroenterology*. 2011; 140:2031–2043. [PubMed: 21419767]
59. Rubtsov YP, et al. Stability of the regulatory T cell lineage in vivo. *Science*. 2010; 329:1667–1671. [PubMed: 20929851]
60. Miyao T, et al. Plasticity of Foxp3(+) T cells reflects promiscuous Foxp3 expression in conventional T cells but not reprogramming of regulatory T cells. *Immunity*. 2012; 36:262–275. [PubMed: 22326580]
61. Hosken NA, Shibuya K, Heath AW, Murphy KM, O'Garra A. The effect of antigen dose on CD4⁺ T helper cell phenotype development in a T cell receptor-alpha beta-transgenic model. *J Exp Med*. 1995; 182:1579–1584. [PubMed: 7595228]
62. Burstein HJ, Abbas AK. In vivo role of interleukin 4 in T cell tolerance induced by aqueous protein antigen. *J Exp Med*. 1993; 177:457–463. [PubMed: 8426114]
63. Yamane H, Zhu J, Paul WE. Independent roles for IL-2 and GATA-3 in stimulating naive CD4⁺ T cells to generate a Th2-inducing cytokine environment. *J Exp Med*. 2005; 202:793–804. [PubMed: 16172258]
64. Iezzi G, Sonderegger I, Ampenberger F, Schmitz N, Marsland BJ, Kopf M. CD40-CD40L cross-talk integrates strong antigenic signals and microbial stimuli to induce development of IL-17-producing CD4⁺ T cells. *Proc Natl Acad Sci USA*. 2009; 106:876–881. [PubMed: 19136631]

65. Fazilleau N, McHeyzer-Williams LJ, Rosen H, McHeyzer-Williams MG. The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding. *Nat Immunol.* 2009; 10:375–384. [PubMed: 19252493]
66. Itoh M, et al. Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol.* 1999; 162:5317–5326. [PubMed: 10228007]
67. Haxhinasto S, Mathis D, Benoist C. The AKT-mTOR axis regulates de novo differentiation of CD4+Foxp3+ cells. *J Exp Med.* 2008; 205:565–574. [PubMed: 18283119]
68. Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, von Boehmer H. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol.* 2005; 6:1219–1227. [PubMed: 16244650]
69. Sauer S, et al. T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR. *Proc Natl Acad Sci USA.* 2008; 105:7797–7802. [PubMed: 18509048]
70. Gottschalk RA, Corse E, Allison JP. TCR ligand density and affinity determine peripheral induction of Foxp3 in vivo. *J Exp Med.* 2010; 207:1701–1711. [PubMed: 20660617]
71. O'Shea JJ, Plenge R. JAK and STAT signaling molecules in immunoregulation and immune-mediated disease. *Immunity.* 2012; 36:542–550. [PubMed: 22520847]
72. O'Shea JJ, Lahesmaa R, Vahedi G, Laurence A, Kanno Y. Genomic views of STAT function in CD4+ T helper cell differentiation. *Nat Rev Immunol.* 2011; 11:239–250. [PubMed: 21436836]
73. Leonard WJ, O'Shea JJ. Jaks and STATs: biological implications. *Annu Rev Immunol.* 1998; 16:293–322. [PubMed: 9597132]
74. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol.* 2010; 28:445–489. [PubMed: 20192806]
75. Shi M, Lin TH, Appell KC, Berg LJ. Janus-kinase-3-dependent signals induce chromatin remodeling at the Ifng locus during T helper 1 cell differentiation. *Immunity.* 2008; 28:763–773. [PubMed: 18549798]
76. van Panhuys N, et al. In vivo studies fail to reveal a role for IL-4 or STAT6 signaling in Th2 lymphocyte differentiation. *Proc Natl Acad Sci USA.* 2008; 105:12423–12428. [PubMed: 18719110]
77. Ben-Sasson SZ, Le Gros G, Conrad DH, Finkelman FD, Paul WE. IL-4 production by T cells from naive donors. IL-2 is required for IL-4 production. *J Immunol.* 1990; 145:1127–1136. [PubMed: 2143207]
78. Cote-Sierra J, et al. Interleukin 2 plays a central role in Th2 differentiation. *Proc Natl Acad Sci USA.* 2004; 101:3880–3885. [PubMed: 15004274]
79. Kagami S, et al. Stat5a regulates T helper cell differentiation by several distinct mechanisms. *Blood.* 2001; 97:2358–2365. [PubMed: 11290598]
80. Zhu J, Cote-Sierra J, Guo L, Paul WE. Stat5 activation plays a critical role in Th2 differentiation. *Immunity.* 2003; 19:739–748. [PubMed: 14614860]
81. Liao W, et al. Priming for T helper type 2 differentiation by interleukin 2-mediated induction of interleukin 4 receptor alpha-chain expression. *Nat Immunol.* 2008; 9:1288–1296. [PubMed: 18820682]
82. Diehl S, et al. Inhibition of Th1 differentiation by IL-6 is mediated by SOCS1. *Immunity.* 2000; 13:805–815. [PubMed: 11163196]
83. Diehl S, Rincon M. The two faces of IL-6 on Th1/Th2 differentiation. *Mol Immunol.* 2002; 39:531–536. [PubMed: 12431386]
84. Stritesky GL, et al. The Transcription Factor STAT3 Is Required for T Helper 2 Cell Development. *Immunity.* 2011; 34:39–49. [PubMed: 21215659]
85. Chen Z, et al. Selective regulatory function of Socs3 in the formation of IL-17-secreting T cells. *Proc Natl Acad Sci USA.* 2006; 103:8137–8142. [PubMed: 16698929]
86. Yang XO, et al. STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J Biol Chem.* 2007; 282:9358–9363. [PubMed: 17277312]

87. Nishihara M, et al. IL-6-gp130-STAT3 in T cells directs the development of IL-17+ Th with a minimum effect on that of Treg in the steady state. *Int Immunol.* 2007; 19:695–702. [PubMed: 17493959]
88. Kimura A, Naka T, Kishimoto T. IL-6-dependent and -independent pathways in the development of interleukin 17-producing T helper cells. *Proc Natl Acad Sci USA.* 2007; 104:12099–12104. [PubMed: 17623780]
89. Harris TJ, et al. Cutting edge: An in vivo requirement for STAT3 signaling in TH17 development and TH17-dependent autoimmunity. *J Immunol.* 2007; 179:4313–437. [PubMed: 17878325]
90. Ma CS, et al. Deficiency of Th17 cells in hyper IgE syndrome due to mutations in STAT3. *J Exp Med.* 2008; 205:1551–1557. [PubMed: 18591410]
91. Milner JD, et al. Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature.* 2008; 452:773–776. [PubMed: 18337720]
92. Peters A, Lee Y, Kuchroo VK. The many faces of Th17 cells. *Curr Opin Immunol.* 2011; 23:702–706. [PubMed: 21899997]
93. Nurieva RI, et al. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity.* 2008; 29:138–149. [PubMed: 18599325]
94. Vogelzang A, McGuire HM, Yu D, Sprent J, Mackay CR, King C. A fundamental role for interleukin-21 in the generation of T follicular helper cells. *Immunity.* 2008; 29:127–137. [PubMed: 18602282]
95. Poholek AC, et al. In vivo regulation of Bcl6 and T follicular helper cell development. *J Immunol.* 2010; 185:313–326. [PubMed: 20519643]
96. Eto D, et al. IL-21 and IL-6 are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4 T cell (Tfh) differentiation. *PloS One.* 2011; 6:e17739. [PubMed: 21423809]
97. Zotos D, et al. IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. *J Exp Med.* 2010; 207:365–378. [PubMed: 20142430]
98. Linterman MA, et al. IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. *J Exp Med.* 2010; 207:353–363. [PubMed: 20142429]
99. Karnowski A, et al. B and T cells collaborate in antiviral responses via IL-6, IL-21, and transcriptional activator and coactivator, Oct2 and OBF-1. *J Exp Med.* 2012; 209:2049–2064. [PubMed: 23045607]
100. Ma CS, et al. Functional STAT3 deficiency compromises the generation of human T follicular helper cells. *Blood.* 2012; 119:3997–4008. [PubMed: 22403255]
101. Ma CS, et al. Early commitment of naive human CD4(+) T cells to the T follicular helper (TFH) cell lineage is induced by IL-12. *Immunol Cell Biol.* 2009; 87:590–600. [PubMed: 19721453]
102. Schmitt N, et al. Human dendritic cells induce the differentiation of interleukin-21-producing T follicular helper-like cells through interleukin-12. *Immunity.* 2009; 31:158–169. [PubMed: 19592276]
103. Laurence A, et al. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity.* 2007; 26:371–381. [PubMed: 17363300]
104. Johnston RJ, Choi YS, Diamond JA, Yang JA, Crotty S. STAT5 is a potent negative regulator of TFH cell differentiation. *J Exp Med.* 2012; 209:243–250. [PubMed: 22271576]
105. Nurieva RI, et al. STAT5 protein negatively regulates T follicular helper (Tfh) cell generation and function. *J Biol Chem.* 2012; 287:11234–11239. [PubMed: 22318729]
106. Ballesteros-Tato A, et al. Interleukin-2 inhibits germinal center formation by limiting T follicular helper cell differentiation. *Immunity.* 2012; 36:847–856. [PubMed: 22464171]
107. Yao Z, et al. Nonredundant roles for Stat5a/b in directly regulating Foxp3. *Blood.* 2007; 109:4368–4375. [PubMed: 17227828]
108. Laurence A, et al. STAT3 transcription factor promotes instability of nTreg cells and limits generation of iTreg cells during acute murine graft-versus-host disease. *Immunity.* 2012; 37:209–222. [PubMed: 22921119]

109. Stumhofer JS, et al. Interleukin 27 negatively regulates the development of interleukin 17-producing T helper cells during chronic inflammation of the central nervous system. *Nat Immunol.* 2006; 7:937–45. [PubMed: 16906166]
110. Hirahara K, et al. Interleukin-27 priming of T cells controls IL-17 production in trans via induction of the ligand PD-L1. *Immunity.* 2012; 36:1017–1030. [PubMed: 22726954]
111. Liu L, et al. Gain-of-function human STAT1 mutations impair IL-17 immunity and underlie chronic mucocutaneous candidiasis. *J Exp Med.* 2011; 208:1635–1648. [PubMed: 21727188]
112. Wei L, et al. Discrete roles of STAT4 and STAT6 transcription factors in tuning epigenetic modifications and transcription during T helper cell differentiation. *Immunity.* 2010; 32:840–851. [PubMed: 20620946]
113. Yang XP, et al. Opposing regulation of the locus encoding IL-17 through direct, reciprocal actions of STAT3 and STAT5. *Nat Immunol.* 2011; 12:247–254. [PubMed: 21278738]
114. Cho JY, Grigura V, Murphy TL, Murphy K. Identification of cooperative monomeric Brachyury sites conferring T-bet responsiveness to the proximal IFN- γ promoter. *Int Immunol.* 2003; 15:1149–1160. [PubMed: 13679385]
115. Sekimata M, et al. CCCTC-binding factor and the transcription factor T-bet orchestrate T helper 1 cell-specific structure and function at the interferon- γ locus. *Immunity.* 2009; 31:551–64. [PubMed: 19818655]
116. Zheng W, Flavell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell.* 1997; 89:587–596. [PubMed: 9160750]
117. Ivanov II, et al. The orphan nuclear receptor ROR γ directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell.* 2006; 126:1121–1133. [PubMed: 16990136]
118. Chang HC, et al. The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. *Nat Immunol.* 2010; 11:527–534. [PubMed: 20431622]
119. Johnston RJ, et al. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science.* 2009; 325:1006–1010. [PubMed: 19608860]
120. Nurieva RI, et al. Bcl6 mediates the development of T follicular helper cells. *Science.* 2009; 325:1001–1005. [PubMed: 19628815]
121. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol.* 2003; 4:330–336. [PubMed: 12612578]
122. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science.* 2003; 299:1057–1061. [PubMed: 12522256]
123. Hwang ES, Szabo SJ, Schwartzberg PL, Glimcher LH. T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3. *Science.* 2005; 307:430–433. [PubMed: 15662016]
124. Koch MA, Tucker-Heard G, Perdue NR, Killebrew JR, Urdahl KB, Campbell DJ. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat Immunol.* 2009; 10:595–602. [PubMed: 19412181]
125. Wang Y, Su MA, Wan YY. An essential role of the transcription factor GATA-3 for the function of regulatory T cells. *Immunity.* 2011; 35:337–348. [PubMed: 21924928]
126. Chung Y, et al. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med.* 2011; 17:983–988. [PubMed: 21785430]
127. Wohlfert EA, et al. GATA3 controls Foxp3(+) regulatory T cell fate during inflammation in mice. *J Clin Invest.* 2011; 121:4503–4315. [PubMed: 21965331]
128. Linterman MA, et al. Foxp3+ follicular regulatory T cells control the germinal center response. *Nat Med.* 2011; 17:975–982. [PubMed: 21785433]
129. Koch MA, Thomas KR, Perdue NR, Smigielski KS, Srivastava S, Campbell DJ. T-bet(+) Treg cells undergo abortive Th1 cell differentiation due to impaired expression of IL-12 receptor β 2. *Immunity.* 2012; 37:501–510. [PubMed: 22960221]
130. Hall AO, et al. The cytokines interleukin 27 and interferon- γ promote distinct Treg cell populations required to limit infection-induced pathology. *Immunity.* 2012; 37:511–523. [PubMed: 22981537]
131. Chaudhry A, et al. CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. *Science.* 2009; 326:986–991. [PubMed: 19797626]

132. Pepper M, Pagan AJ, Igyarto BZ, Taylor JJ, Jenkins MK. Opposing signals from the Bcl6 transcription factor and the interleukin-2 receptor generate T helper 1 central and effector memory cells. *Immunity*. 2011; 35:583–595. [PubMed: 22018468]
133. Oestreich KJ, Huang AC, Weinmann AS. The lineage-defining factors T-bet and Bcl-6 collaborate to regulate Th1 gene expression patterns. *J Exp Med*. 2011; 208:1001–1013. [PubMed: 21518797]
134. Oestreich KJ, Mohn SE, Weinmann AS. Molecular mechanisms that control the expression and activity of Bcl-6 in TH1 cells to regulate flexibility with a TFH-like gene profile. *Nat Immunol*. 2012; 13:405–411. [PubMed: 22406686]
135. Zhang JA, Mortazavi A, Williams BA, Wold BJ, Rothenberg EV. Dynamic transformations of genome-wide epigenetic marking and transcriptional control establish T cell identity. *Cell*. 2012; 149:467–482. [PubMed: 22500808]
136. Xiong Y, Bosselut R. CD4-CD8 differentiation in the thymus: connecting circuits and building memories. *Curr Opin Immunol*. 2012; 24:139–145. [PubMed: 22387323]
137. Zheng Y, et al. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. *Nature*. 2009; 458:351–356. [PubMed: 19182775]
138. Ahyi AN, Chang HC, Dent AL, Nutt SL, Kaplan MH. IFN regulatory factor 4 regulates the expression of a subset of Th2 cytokines. *J Immunol*. 2009; 183:1598–1606. [PubMed: 19592658]
139. Staudt V, et al. Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells. *Immunity*. 2010; 33:192–202. [PubMed: 20674401]
140. Brustle A, et al. The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. *Nat Immunol*. 2007; 8:958–966. [PubMed: 17676043]
141. Kwon H, et al. Analysis of interleukin-21-induced Prdm1 gene regulation reveals functional cooperation of STAT3 and IRF4 transcription factors. *Immunity*. 2009; 31:941–952. [PubMed: 20064451]
142. Glasmacher E, et al. A genomic regulatory element that directs assembly and function of immune-specific AP-1-IRF complexes. *Science*. 2012; 338:975–980. [PubMed: 22983707]
143. Li P, et al. BATF-JUN is critical for IRF4-mediated transcription in T cells. *Nature*. 2012; 490:543–546. [PubMed: 22992523]
144. Ciofani M, et al. A validated regulatory network for th17 cell specification. *Cell*. 2012; 151:289–303. [PubMed: 23021777]
145. Ho IC, Hodge MR, Rooney JW, Glimcher LH. The proto-oncogene c-maf is responsible for tissue-specific expression of interleukin-4. *Cell*. 1996; 85:973–983. [PubMed: 8674125]
146. Bauquet AT, et al. The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. *Nature immunology*. 2009; 10:167–175. [PubMed: 19098919]
147. Mullen AC, et al. Hlx is induced by and genetically interacts with T-bet to promote heritable TH1 gene induction. *Nat Immunol*. 2002; 3:652–658. [PubMed: 12055627]
148. Djuretic IM, Levanon D, Negreanu V, Groner Y, Rao A, Ansel KM. Transcription factors T-bet and Runx3 cooperate to activate Ifng and silence Il4 in T helper type 1 cells. *Nat Immunol*. 2007; 8:145–153. [PubMed: 17195845]
149. Grenningloh R, Kang BY, Ho IC. Ets-1, a functional cofactor of T-bet, is essential for Th1 inflammatory responses. *J Exp Med*. 2005; 201:615–626. [PubMed: 15728239]
150. Li B, Tournier C, Davis RJ, Flavell RA. Regulation of IL-4 expression by the transcription factor JunB during T helper cell differentiation. *EMBO J*. 1999; 18:420–432. [PubMed: 9889198]
151. Zhu J, et al. Growth factor independent-1 induced by IL-4 regulates Th2 cell proliferation. *Immunity*. 2002; 16:733–744. [PubMed: 12049724]
152. Dang EV, et al. Control of T(H)17/T(reg) balance by hypoxia-inducible factor 1. *Cell*. 2011; 146:772–784. [PubMed: 21871655]
153. Lazarevic V, et al. T-bet represses T(H)17 differentiation by preventing Runx1-mediated activation of the gene encoding RORgammat. *Nat Immunol*. 2011; 12:96–104. [PubMed: 21151104]

154. Quintana FJ, et al. Aiolos promotes TH17 differentiation by directly silencing IL2 expression. *Nat Immunol.* 2012; 13:770–777. [PubMed: 22751139]
155. Hamalainen H, Zhou H, Chou W, Hashizume H, Heller R, Lahesmaa R. Distinct gene expression profiles of human type 1 and type 2 T helper cells. *Genome Biol.* 2001; 2 RESEARCH0022.
156. Rogge L, et al. Transcript imaging of the development of human T helper cells using oligonucleotide arrays. *Nat Genet.* 2000; 25:96–101. [PubMed: 10802665]
157. Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods.* 2008; 5:621–628. [PubMed: 18516045]
158. Liston A, Lu LF, O'Carroll D, Tarakhovsky A, Rudensky AY. Dicer-dependent microRNA pathway safeguards regulatory T cell function. *J Exp Med.* 2008; 205:1993–2004. [PubMed: 18725526]
159. Zhou X, et al. Selective miRNA disruption in T reg cells leads to uncontrolled autoimmunity. *J Exp Med.* 2008; 205:1983–1991. [PubMed: 18725525]
160. Koralov SB, et al. Dicer ablation affects antibody diversity and cell survival in the B lymphocyte lineage. *Cell.* 2008; 132:860–874. [PubMed: 18329371]
161. Kuchen S, et al. Regulation of microRNA expression and abundance during lymphopoiesis. *Immunity.* 2010; 32:828–839. [PubMed: 20605486]
162. Rossi RL, et al. Distinct microRNA signatures in human lymphocyte subsets and enforcement of the naive state in CD4+ T cells by the microRNA miR-125b. *Nat Immunol.* 2011; 12:796–803. [PubMed: 21706005]
163. Stittrich AB, et al. The microRNA miR-182 is induced by IL-2 and promotes clonal expansion of activated helper T lymphocytes. *Nat Immunol.* 2010; 11:1057–1062. [PubMed: 20935646]
164. Takahashi H, et al. TGF- β and retinoic acid induce the microRNA miR-10a, which targets Bcl-6 and constrains the plasticity of helper T cells. *Nat Immunol.* 2012; 13:587–595. [PubMed: 22544395]
165. Rinn JL, Chang HY. Genome regulation by long noncoding RNAs. *Annu Rev Biochem.* 2012; 81:145–166. [PubMed: 22663078]
166. Barlow DP, Stoger R, Herrmann BG, Saito K, Schweifer N. The mouse insulin-like growth factor type-2 receptor is imprinted and closely linked to the Tme locus. *Nature.* 1991; 349:84–87. [PubMed: 1845916]
167. Brown CJ, et al. A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome. *Nature.* 1991; 349:38–44. [PubMed: 1985261]
168. Collier SP, Collins PL, Williams CL, Boothby MR, Aune TM. Cutting edge: Influence of Tmevpg1, a long intergenic noncoding RNA, on the expression of Ifng by Th1 cells. *J Immunol.* 2012; 189:2084–2088. [PubMed: 22851706]
169. ENCODE. An integrated encyclopedia of DNA elements in the human genome. *Nature.* 2012; 489:57–74. [PubMed: 22955616]
170. Amsen D, Spilianakis CG, Flavell RA. How are T(H)1 and T(H)2 effector cells made? *Curr Opin Immunol.* 2009; 21:153–160. [PubMed: 19375293]
171. Aune TM, Collins PL, Chang S. Epigenetics and T helper 1 differentiation. *Immunology.* 2009; 126:299–305. [PubMed: 19178593]
172. Wilson CB, Rowell E, Sekimata M. Epigenetic control of T-helper-cell differentiation. *Nat Rev Immunol.* 2009; 9:91–105. [PubMed: 19151746]
173. Ansel KM, Lee DU, Rao A. An epigenetic view of helper T cell differentiation. *Nat Immunol.* 2003; 4:616–623. [PubMed: 12830136]
174. Kanno Y, Vahedi G, Hirahara K, Singleton K, O'Shea JJ. Transcriptional and epigenetic control of T helper cell specification: molecular mechanisms underlying commitment and plasticity. *Annu Rev Immunol.* 2012; 30:707–731. [PubMed: 22224760]
175. Oestreich KJ, Weinmann AS. Encoding stability versus flexibility: lessons learned from examining epigenetics in T helper cell differentiation. *Curr Top Microbiol Immunol.* 2012; 356:145–164. [PubMed: 21748629]

176. Zhang F, Boothby M. T helper type 1-specific Brg1 recruitment and remodeling of nucleosomes positioned at the IFN- γ promoter are Stat4 dependent. *J Exp Med*. 2006; 203:1493–1505. [PubMed: 16717115]
177. Kagey MH, et al. Mediator and cohesin connect gene expression and chromatin architecture. *Nature*. 2010; 467:430–435. [PubMed: 20720539]
178. Hadjur S, et al. Cohesins form chromosomal cis-interactions at the developmentally regulated IFNG locus. *Nature*. 2009; 460:410–U130. [PubMed: 19458616]
179. Yamashita M, et al. Crucial role of MLL for the maintenance of memory T helper type 2 cell responses. *Immunity*. 2006; 24:611–622. [PubMed: 16713978]
180. Onodera A, et al. STAT6-mediated displacement of polycomb by trithorax complex establishes long-term maintenance of GATA3 expression in T helper type 2 cells. *J Exp Med*. 2010; 207:2493–2506. [PubMed: 20956546]
181. Allan RS, et al. An epigenetic silencing pathway controlling T helper 2 cell lineage commitment. *Nature*. 2012; 487:249–253. [PubMed: 22763435]
182. Kim HP, Leonard WJ. CREB/ATF-dependent T cell receptor-induced FoxP3 gene expression: a role for DNA methylation. *J Exp Med*. 2007; 204:1543–1551. [PubMed: 17591856]
183. Floess S, et al. Epigenetic control of the foxp3 locus in regulatory T cells. *PLoS Biology*. 2007; 5:e38. [PubMed: 17298177]
184. Huehn J, Polansky JK, Hamann A. Epigenetic control of FOXP3 expression: the key to a stable regulatory T-cell lineage? *Nat Rev Immunol*. 2009; 9:83–89. [PubMed: 19114986]
185. Xu L, Kitani A, Stuelten C, McGrady G, Fuss I, Strober W. Positive and negative transcriptional regulation of the Foxp3 gene is mediated by access and binding of the Smad3 protein to enhancer I. *Immunity*. 2010; 33:313–325. [PubMed: 20870174]
186. Park PJ. ChIP-seq: advantages and challenges of a maturing technology. *Nat Rev Genet*. 2009; 10:669–680. [PubMed: 19736561]
187. Wang YH, et al. A novel subset of CD4(+) T(H)2 memory/effector cells that produce inflammatory IL-17 cytokine and promote the exacerbation of chronic allergic asthma. *J Exp Med*. 2010; 207:2479–2491. [PubMed: 20921287]
188. Durant L, et al. Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis. *Immunity*. 2010; 32:605–615. [PubMed: 20493732]
189. Hoey T, et al. Distinct requirements for the naturally occurring splice forms Stat4alpha and Stat4beta in IL-12 responses. *EMBO J*. 2003; 22:4237–4248. [PubMed: 12912921]
190. Lund RJ, Chen Z, Scheinin J, Lahesmaa R. Early target genes of IL-12 and STAT4 signaling in th cells. *J Immunol*. 2004; 172:6775–6782. [PubMed: 15153495]
191. Elo LL, et al. Genome-wide profiling of interleukin-4 and STAT6 transcription factor regulation of human Th2 cell programming. *Immunity*. 2010; 32:852–862. [PubMed: 20620947]
192. Zheng Y, Josefowicz SZ, Kas A, Chu TT, Gavin MA, Rudensky AY. Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. *Nature*. 2007; 445:936–940. [PubMed: 17237761]
193. Ivanov II, Zhou L, Littman DR. Transcriptional regulation of Th17 cell differentiation. *Semin Immunol*. 2007; 19:409–417. [PubMed: 18053739]
194. Ramana CV, Chatterjee-Kishore M, Nguyen H, Stark GR. Complex roles of Stat1 in regulating gene expression. *Oncogene*. 2000; 19:2619–2627. [PubMed: 10851061]
195. Korn T, et al. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature*. 2007; 448:484–487. [PubMed: 17581588]
196. Yang XO, et al. T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity*. 2008; 28:29–39. [PubMed: 18164222]
197. Miller SA, Weinmann AS. Molecular mechanisms by which T-bet regulates T-helper cell commitment. *Immunol Rev*. 2010; 238:233–246. [PubMed: 20969596]
198. Beima KM, Miazgowicz MM, Lewis MD, Yan PS, Huang TH, Weinmann AS. T-bet binding to newly identified target gene promoters is cell type-independent but results in variable context-dependent functional effects. *J Biol Chem*. 2006; 281:11992–2000. [PubMed: 16473879]

199. Nguyen KB, et al. Critical role for STAT4 activation by type 1 interferons in the interferon-gamma response to viral infection. *Science*. 2002; 297:2063–2066. [PubMed: 12242445]
200. Kao C, et al. Transcription factor T-bet represses expression of the inhibitory receptor PD-1 and sustains virus-specific CD8+ T cell responses during chronic infection. *Nat Immunol*. 2011; 12:663–671. [PubMed: 21623380]
201. Zhu J, et al. The transcription factor T-bet is induced by multiple pathways and prevents an endogenous Th2 cell program during Th1 cell responses. *Immunity*. 2012; 37:660–673. [PubMed: 23041064]
202. Wei G, et al. Genome-wide analyses of transcription factor GATA3-mediated gene regulation in distinct T cell types. *Immunity*. 2011; 35:299–311. [PubMed: 21867929]
203. Samstein RM, et al. Foxp3 exploits a pre-existent enhancer landscape for regulatory T cell lineage specification. *Cell*. 2012; 151:153–166. [PubMed: 23021222]
204. Gavin MA, et al. Foxp3-dependent programme of regulatory T-cell differentiation. *Nature*. 2007; 445:771–775. [PubMed: 17220874]
205. Rudra D, et al. Transcription factor Foxp3 and its protein partners form a complex regulatory network. *Nat Immunol*. 2012; 13:1010–1019. [PubMed: 22922362]
206. McMurchy AN, et al. A novel function for FOXP3 in humans: intrinsic regulation of conventional T cells. *Blood*. 2012 in press.
207. Rudensky AY. Regulatory T cells and Foxp3. *Immunol Rev*. 2011; 241:260–268. [PubMed: 21488902]
208. Basso K, Dalla-Favera R. BCL6: master regulator of the germinal center reaction and key oncogene in B cell lymphomagenesis. *Adv Immunol*. 2010; 105:193–210. [PubMed: 20510734]
209. Arima M, et al. A putative silencer element in the IL-5 gene recognized by Bcl6. *J Immunol*. 2002; 169:829–836. [PubMed: 12097386]
210. Crotty S, Johnston RJ, Schoenberger SP. Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. *Nat Immunol*. 2010; 11:114–120. [PubMed: 20084069]
211. Yu D, et al. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity*. 2009; 31:457–468. [PubMed: 19631565]
212. Ansel KM, Djuretic I, Tanasa B, Rao A. Regulation of Th2 differentiation and Il4 locus accessibility. *Annu Rev Immunol*. 2006; 24:607–656. [PubMed: 16551261]
213. Harada Y, et al. The 3' enhancer CNS2 is a critical regulator of interleukin-4-mediated humoral immunity in follicular helper T cells. *Immunity*. 2012; 36:188–200. [PubMed: 22365664]
214. Spilianakis CG, Flavell RA. Long-range intrachromosomal interactions in the T helper type 2 cytokine locus. *Nat Immunol*. 2004; 5:1017–1027. [PubMed: 15378057]
215. Samstein RM, Josefowicz SZ, Arvey A, Treuting PM, Rudensky AY. Extrathymic generation of regulatory T cells in placental mammals mitigates maternal-fetal conflict. *Cell*. 2012; 150:29–38. [PubMed: 22770213]
216. Vahedi G, et al. STATs shape the active enhancer landscape of T cell populations. *Cell*. 2012; 151:981–993. [PubMed: 23178119]
217. Mercer E, et al. Multilineage priming of enhancer repertoires precedes commitment to the B and myeloid cell lineages in hematopoietic progenitors. *Immunity*. 2011; 35:413–425. [PubMed: 21903424]
218. Biddie S, et al. Transcription factor AP1 potentiates chromatin accessibility and glucocorticoid receptor binding. *Molecular Cell*. 2011; 43:145–155. [PubMed: 21726817]

“Classical” model of the role of master regulators for CD4 T cell differentiation

Fig. 1. ‘Classical’ model of the role of master regulators for CD4⁺ T-cell differentiation
 From the classical monolithic perspective, each helper cell subset expresses a lineage-defining ‘master regulator’ transcription factor, which is necessary and sufficient to induce each subsets specific gene-expression.

Fig. 2. Models for co-expression of master regulators

Recent advances have argued for a more nuanced view of master regulators. Abundant data indicate that helper cells often express more than one master regulator. In this regard, one can view expression of master regulators in three ways. (A) “Subspecialization” model. Co-expression of two master regulators is functionally important for Foxp3⁺ Treg cells. The functional responses of Treg cells can be specialized or targeted based on the expression of a master regulator co-expressed with Foxp3⁺. Each sub-population shows the specific repressive activity for each helper T-cell subset. (B) ‘Flexibility’ model. Recent studies have also revealed greater flexible behavior of helper T-cell subsets. Epigenetic modifications facilitate flexible co-expression of transcription factors, and consequently the co-expression

of non-canonical cytokines. In principle, such a scenario permits flexible responses to offending pathogens, in this way host defense may be maximized. (C) ‘Balanced (Competition)’ model. In the case of T-bet and Bcl6, these factors are co-expressed, but are functionally antagonistic. From this perspective, responses are constrained or tuned in response to exogenous factors. In reality, it is not just two factors that are regulated but probably dozens. To truly understand the complexity of helper T-cell specification, it is more appropriate to think about highly regulated expression of networks of transcription factors responding to exogenous and endogenous signals.

Fig. 3. Model for the role of pioneering factors and master regulators during CD4⁺ T-cell differentiation

Various perturbations like TCR occupancy or the cytokine milieu can induce ‘pioneering factors’ and activate different STATs, which are indispensable for establishing the genomic epigenetic landscapes of developing helper T cells and dictating the accessibility of key target genes. New data argue that helper T-cell master regulators do not have major effects on the chromatin organization of helper T cells. On the contrary, they have focused actions on key genes which are crucial for lineage specificity or limit alternative fates. Helper T-cell master regulators may be better viewed as critical modulators but not drivers of the chromatin architecture underlying helper cell identity.