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Review Article

Transcriptional and Epigenetic Regulation of Context-Dependent Plasticity in T-Helper Lineages

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Conflict of Interest
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ABSTRACT

Th cell lineage determination and functional specialization are tightly linked to the activation of lineage-determining transcription factors (TFs) that bind *cis*-regulatory elements. These lineage-determining TFs act in concert with multiple layers of transcriptional regulators to alter the epigenetic landscape, including DNA methylation, histone modification and three-dimensional chromosome architecture, in order to facilitate the specific Th gene expression programs that allow for phenotypic diversification. Accumulating evidence indicates that Th cell differentiation is not as rigid as classically held; rather, extensive phenotypic plasticity is an inherent feature of T cell lineages. Recent studies have begun to uncover the epigenetic programs that mechanistically govern T cell subset specification and immunological memory. Advances in next generation sequencing technologies have allowed global transcriptomic and epigenomic interrogation of CD4+ Th cells that extends previous findings focusing on individual loci. In this review, we provide an overview of recent genome-wide insights into the transcriptional and epigenetic regulation of CD4+ T cell-mediated adaptive immunity and discuss the implications for disease as well as immunotherapies.

Keywords: Helper T-lymphocytes; Cell plasticity; Epigenomics; Cancer; Inflammation; Cellular microenvironment

INTRODUCTION

Transcriptional and epigenetic regulation plays a pivotal role during lineage determination. During the process of differentiation, lineage-specific genes are induced while genes of other lineages are silenced. The changes in gene expression are primarily regulated by transcriptional mechanisms through the binding of transcription factors (TFs) to *cis*-regulatory elements (CREs) in the genome, including proximal promoters and distal regulatory enhancers that precisely eukaryotic transcription through their physical interaction over large genomic distances (1). The position and accessibility of these CREs are controlled by epigenetic mechanisms, such as DNA methylation status, nucleosome

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Abbreviations

3C, chromosome conformation capture; 3D, three-dimensional; 4C, circular chromosome conformation capture; 5-azaC, 5-azacytidine; 5-aza-dC, 5-aza-2′-deoxycytidine; 5hmC, 5-hydroxymethylcytosine; 5mc, 5-methyltcytosine; ALS, amyotrophic lateral sclerosis; AML, acute myelogenous leukemia; asRNA, antisense RNA; ATAC-seq, assay for transposase-accessible chromatin using sequencing; BCL6, B-cell lymphoma 6; BET, bromodomain and extra-terminal domain; CAR, chimeric antigen receptor; caRNA-seq, chromatin-associated RNA sequencing; cART, combined antiretroviral therapy; CGI, CpG island; CGRE, GATA response element; ChEC-seq, chromatin endogenous cleavage sequencing; ChIP-seq, chromatin immunoprecipitation sequencing; CLPs, commom lymphoid progenitors; CNSs, conserved non-coding sequences; CRE, cis-regulatory element; CRISPRa, CRISPR-mediated activation; CRISPRi, CRISPR-mediated interference; CTCF, CCCTC-binding factor; CTCL, cutaneous T cell lymphoma; DamID, DNA adenine methyltransferase identification; DBD, DNA-binding domain; dCas9, deactivated Cas9; DHSs, DNase I hypersensitive sites; DN, double-negative; DNase-seq, DNase I hypersensitive sites sequencing; DNMT, DNA methyltransferase; DP, double-positive; EM-seq, enzymatic methyl-seq; E-P, enhancer-promoter; eRNA, enhancer RNA; ERV, endogenous retrovirus; FAIRE-seq, formaldehyde-assisted isolation of regulatory element; HD, Huntington's disease; HDAC, histone deacetylase; HDACi, HDAC inhibitor; HDR, homology-directed repair; HMA, hypomethylating agent; IDH1/2, isocitrate dehydrogenase 1 and 2; iNKT cells, invariant natural killer T cells; itCHIP-seq, indexing and tagmentation-based chromatin immunoprecipitation sequencing; ITK, inducible T cell kinase; KAS-seq, N3-kethoxal-assisted ssDNA sequencing; KDM, multiple lysine demethylase; LBD, ligand-binding domain; LCR, locus control region; lncRNA, long non coding RNA; MDS, myelodysplastic syndrome; MHC, major histocompatibility complex; miRNAS, micro RNA; MNase-seq, micrococcal nuclease digestion with deep sequencing; NAD, nicotinamide adenine dinucleotide; ncRNA, non-coding RNA; NET-seq, native elongating transcript sequencing; NGS, next generation sequencing; NR, nuclear receptor; PARP, poly(ADP-ribose) polymerase; PcG, Polycomb group; PLAC-seq, proximity ligation-assisted chromatin

remodeling/repositioning, and post-translational modification (PTM) of histones as well as other chromatin-associated factors. These epigenetic changes affect the transcriptional regulation of gene expression not only by impacting TF binding but also by modulating three-dimensional (3D) chromatin organization, which includes topologically associated domains (TADs) and enhancer-promoter (E-P) interactions (2).

The development, differentiation, function and plasticity of immune cells, including effector T cells, are also tightly regulated through intrinsic epigenetic mechanisms. Studies utilizing DNA methylation-sensitive or -insensitive restriction enzymes to assess the epigenetic status of loci in Th cell subsets first reported epigenetic regulation of T cell development more than two decades ago (3). The introduction of molecular biology techniques coupled with next-generation sequencing (NGS) technology has dramatically elevated our appreciation of epigenetic regulation in T cells. NGS-derived epigenome maps have revealed that CD4+ T cells undergo extensive changes in DNA methylation, nucleosome remodeling, histone modification and 3D chromatin structure during development and differentiation toward each Th cells lineage in response to various immune stimuli. Furthermore, application of single cell RNA sequencing to interrogate the microenvironments of various immune-related diseases and cancers has shown that effector CD4+ T cells can re-polarize towards a mixed fate and exhibit phenotypic plasticity in response to changing environmental contexts. This research has yielded genome-wide T cell epigenetic profiles and has identified the enzymes directly engaged in chromatin remodeling, providing the field with a deeper understanding of T cell biology, including how T cells guard against a variety of diseases.

These discoveries have also enabled the creation of novel diagnostic and therapeutic approaches to treat and even cure diseases that were previously invariably fatal. Recent and significant advancements in cancer immunotherapy provide a prime illustration of how the specificity and endurance of T-cell immune responses can be leveraged (4). These include the introduction of therapeutics that rely on Ab blockage of T cell inhibitory receptors to their corresponding ligand and adoptive T cell therapy with unedited or chimeric antigen receptors (CAR)-expressing T cells (5). While these approaches have dramatically improved the treatment options for numerous malignancies, there is a substantial need for further optimization. Notably, accumulating evidence indicates that the durability of T-cell-elicited immune responses depends on mechanisms governing cell fate commitment (6).

Here, we seek to provide an informative summary of the literature explaining the mechanisms involved in the epigenetic regulation of T cell differentiation, focusing on the activation of lineage-determinant TFs and their control of key genes in each Th subset. In addition, we will summarize current genome-wide approaches to investigate the epigenetic landscape and discuss the implications of our current understanding of the Th-cell epigenome for improving existing and future therapies.

TECHNOLOGIES FOR INVESTIGATING EPIGENOMIC PROFILES

In this review, we have embraced a broad definition of the term "epigenetics" to include any changes in phenotype that are genomically derived without an alteration in genotype (7). Accordingly, epigenetic changes can include chemical modification of DNA or RNA bases, nucleosome remodeling, posttranslational modification of histones, altered expression or

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immunoprecipitation sequencing; PRO-seq, precision run-on sequencing; PTCL, peripheral T-cell lymphoma; PTM, post-translational modification; Rore, ROR response element; RRBS, reduced representation bisulfite sequencing; SAHA, suberoylanilide hydroxamic acid; SAM, S-adenosylmethionine; scRNA-seq, single cell RNA sequencing; SD1, lysine demethylase 1; SP, single positive; STARR-seq, self-transcribing active regulatory region sequencing; TAD, topplogical associate domain; TCM, central memory T; TCR, T-cell receptor; TEC, regulatory T cell; TF, transcrition factor; Tfh, follicular helper T cell; Th, cell T helper cell; TIME, tumor immune microenvironment; Treg, regulatory T cell; TrxG, trithorax group; TSS, transcription start site; TTS, transcription termination site; TT-seq, transient transcriptome sequencing; UMI, unique molecular identifier; WGBS, whole genome bisulfite sequencing.

Author Contributions

Conceptualization: Oh S, Lee JY; Funding acquisition: Oh S, Lee JY; Supervision: Oh S, Lee JY; Visualization: Lee H; Writing – original draft: Friedman MJ, Oh S, Lee JY, Lee H; Writing - review & editing: Friedman MJ, Oh S, Lee JY, Lee H.

chromatin localization of non-coding RNAs (ncRNAs), and adjustments to 3D chromatin organization (8). Various methods coupled with NGS technology are now widely used for studying epigenetic changes in an unbiased, genome-wide manner.

DNA methylation

The most prevalent DNA modification is the methylation of cytosine to yield 5-methylcytosine (5mC) by the DNA methyltransferases (DNMT) family of DNA methyltransferases at CpG dinucleotide sequences (9). CpG methylation is generally correlated with gene repression, as exemplified in X chromosome inactivation (10). Treatment of genomic DNA with sodium bisulfite results in deamination of unmethylated cytosines to uracil while leaving 5mC intact. By converting cytosine to uracil, methyl-seq strategies, such as whole genome bisulfite sequencing (WGBS), allow unmethylated versus methylated cytosine to be distinguished at single base-pair resolution (11) (**Fig. 1A**). Although sequencing of bisulfite-converted genomic DNA is regarded as a gold standard for methylated DNA detection, the approach is expensive and, due to DNA degradation caused by the harshness of bisulfite treatment, there is a requirement for large input amounts. To overcome this technical pitfall, enzymatic methyl-seq (EM-seq) was developed. EM-seq involves sequential enzymatic reactions: a TET2 reaction for further modification of 5mC followed by an APOBEC reaction for deamination of unmethylated cytosines to uracil (12) (**Fig. 1A**). For cost-effective detection of 5mC, approaches that enrich the genomic sequences of interest either by oligonucleotide hybridization (13) or by digestion with a restriction enzyme that recognizes CCGG motifs (reduced representation bisulfite sequencing, or RRBS) (14) have been developed.

DNA accessibility

Eukaryotic chromatin is composed of nucleosomes, which is the basic unit of chromatin structure consisting of a histone octamer wrapped by 147bp of DNA. The nucleosome density determines the accessibility of chromatin. Tightly packaged, closed DNA regions have dense nucleosomes while DNA regions with fewer nucleosomes are more accessible. Because chromatin accessibility is highly correlated with gene expression and displays cell type specificity, several NGS methods to assess chromatin accessibility in a genome-wide manner have been developed, such as micrococcal nuclease digestion with deep sequencing (MNase-seq), DNase I hypersensitive sites sequencing (DNase-seq), formaldehyde-assisted isolation of regulatory elements (FAIRE-seq) and assay for transposase-accessible chromatin with sequencing (ATAC-seq) (15) (**Fig. 1B**). MNase-seq is based on the endo- and exo-nuclease activity of MNase which can digest DNA that is not protected by nucleosomes or TFs binding. Therefore, MNase-seq measures nucleosome-occupied regions that are regarded as closed chromatin. DNase-seq and FAIRE-seq detect nucleosome-free regions by using endonuclease DNase I and sonication, respectively. In the presence of a low concentration of DNase I, there is preferential digestion of nucleosome-free genomic regions, which are characterized as DNase I hypersensitive sites (DHSs). For FAIRE-seq, formaldehyde-fixed chromatin is sonicated and then separated by phenol-chloroform extraction, with nucleosome-free DNA fragments preferentially located in the aqueous phase. ATAC-seq involves 'tagmentation,' or Tn5 transposase-mediated insertion of sequencing adaptors into open chromatin. To date, ATAC-seq has been the primary method employed for genome-wide chromatin accessibility measurements due to its high sensitivity, requirement for limited cell numbers (i.e., single-cell assays), and the convenience of sample preparation. The regulated chromatin accessibility landscape during T-cell lineage determination and commitment of each Th cell subtype, including Th1, Th2, Th17, Tfh and Treg subsets, have been studied comprehensively by using ATAC-seq (16-20).

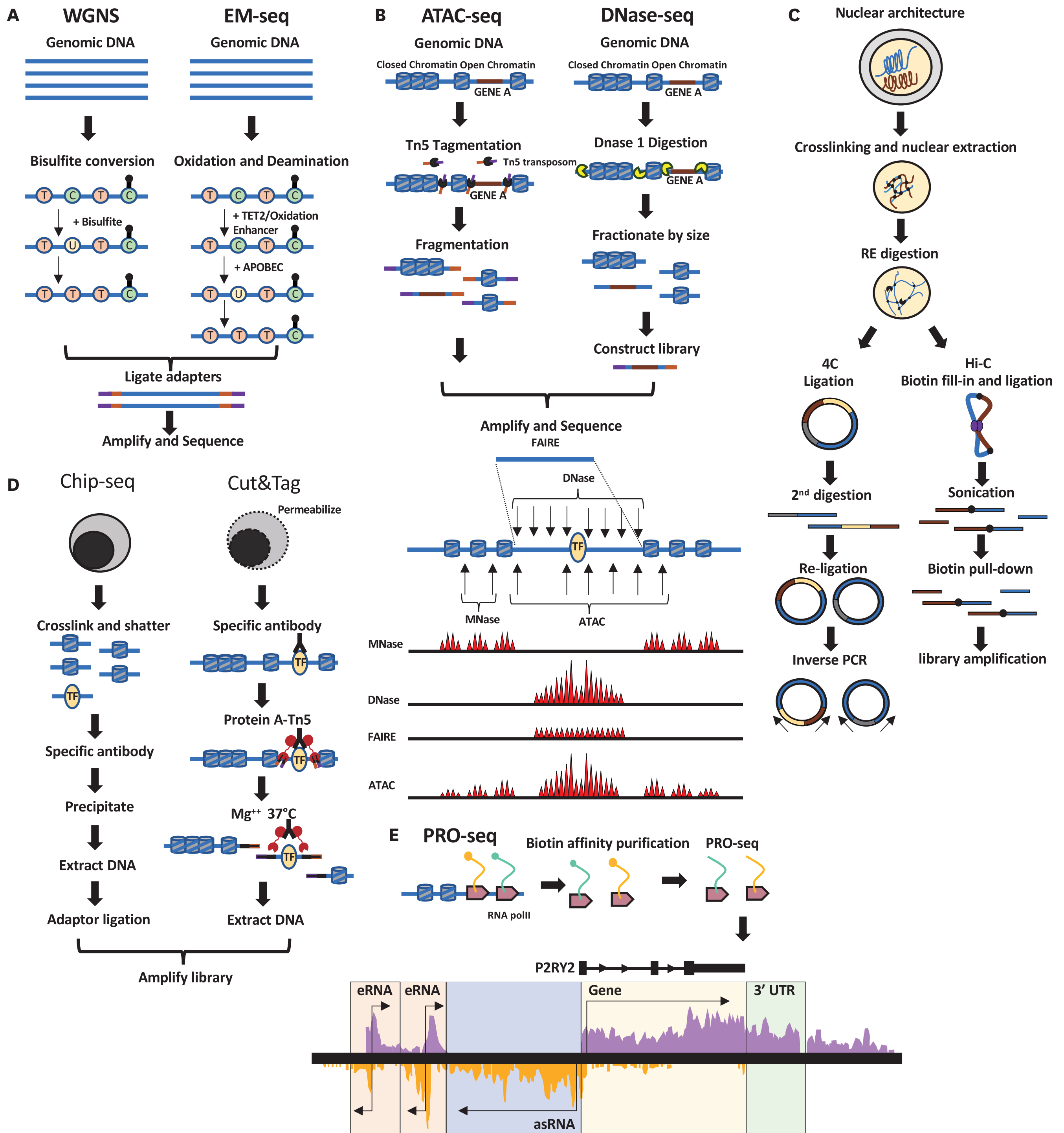

Figure 1. Schematic representation of assays for studying the epigenetic and transcriptional landscape. (A) WGBS and EM-seq: NGS methods for identifying methylated cytosine by converting unmethylated cytosine to uracil. (B) ATAC-seq and DNase-seq: NGS methods for analyzing genome-wide chromatin accessibility by fragmenting DNA. The graph below is a schematic diagram of the peaks generated by each chromatin accessibility analysis as indicated. (C) ChIP-seq and CUT&Tag: NGS methods for analyzing the interaction between protein and DNA by determining the DNA binding sites of a specific protein. (D) PRO-seq: NGS method for interrogating nascent RNA by *in vitro* run-on with biotin-labeled nucleotides. The graph below is a genome browser schematic displaying the PRO-seq profile at a specific locus. eRNA, asRNA, and gene transcription are detected as indicated. (E) Hi-C and chromosome conformation capture-on-chip/circular chromosome conformation capture (4C-seq): NGS methods for assessing chromatin conformation through DNA crosslinking.

Histone modification and TF binding

Current knowledge of the epigenomic landscape largely relies on Ab-based pulldown assays, namely chromatin immunoprecipitation with sequencing (ChIP-seq) (21) and various refinements of the technique. Although massive parallel sequencing of ChIP DNA provides the genome-wide distribution of post-translational histone modifications, TF binding sites, and DNA or RNA modifications, the application is often limited by the requirement of a large number of cells, high background attributable to standard formaldehyde fixation, and potential epitope destruction due to extensive sonication. Alternatives to ChIP-seq using enzymatic activities, such as DamID, ChEC-seq, itChIP-seq, CUT&RUN, and CUT&Tag, were developed to overcome these limitations. Recently, CUT&RUN and CUT&Tag have emerged as the more robust and accessible alternatives to ChIP-seq (22). Although CUT&RUN and CUT&Tag still require specific Ab binding to the target, unlike ChIP, they use unfixed or briefly fixed cells and an enzymatic reaction mediated by protein A-conjugated MNase and Tn5, respectively, instead of sonication. Accordingly, these methods can deliver greatly enhanced signal-to-noise. In addition, most Abs suitable for immunofluorescence staining can be employed in CUT&RUN or CUT&Tag, irrespective of their performance in ChIP, increasing available options (23). Furthermore, library preparation for CUT&Tag is simplified by Ab-targeted tagmentation, allowing for its application in single-cell experiments and the simultaneous profiling of multiple chromatin factors in the same sample (24) (**Fig.1c**). Despite concerns about Tn5 enzyme bias, CUT&Tag has obvious advantages: it is simple, relatively inexpensive, given that it requires low cell numbers and low sequencing depth, and is amenable to further adaptation. To be sure, CUT&Tag should enable future studies to unveil the context-dependent plasticity of immune cells.

Non-coding RNA

Accumulating evidence suggests that ncRNAs, such as long non-coding RNAs (lncRNAs), micro RNAs (miRNAs), antisense RNAs (asRNAs) and enhancer RNAs (eRNAs), have mechanistic roles in a range of cellular activities or features, including gene expression, chromatin organization, RNA stability, and splicing. A number of ncRNAs have been shown to affect Th cell differentiation and function. For example, the Th1-specific lncRNA Linc-MAF-4 suppresses expression of the Th2 TF MAF by interacting with chromatin modifier proteins EZH2 and lysine demethylase 1 (LSD1) to elicit promoter deposition of the repressive histone mark H3K27me3 (25). In addition, many miRNAs are known to influence Th cell function. For example, miR-125b promotes Treg cell differentiation and suppresses Th17 specification while miR-210 enhances Th17 and Th1 responses but inhibits Th2 polarization (the role of miRNAs in Th cells has been reviewed elsewhere (26)). asRNAs, which are transcribed in the opposite direction from protein-coding gene promoters, also have been ascribed functions. IFNG-AS1 and GATA3-AS1 can augment their associated Th phenotypes, Th1 and Th2, respectively (27).

In humans and mice, estimates for ncRNA genes (~100,000) far exceed those for protein-coding genes (28). A large portion of lncRNAs harbors a 5’ cap and poly-A tail, allowing for their detection in a genome-wide manner by conventional poly(A)- or ribo-depletion-based RNA-seq. However, other non-coding RNAs, including miRNAs, asRNAs, and eRNAs, generally require special sequencing methods due to a lack of poly(A) tail and/or limited stability. For miRNA detection, the small RNA-seq method is widely used that has modifications meant to reduce intrinsic bias toward small-sized RNAs (29). Briefly, the small RNA-seq method is composed of a three-parts, including size selection of small RNAs from total RNA, 3’ and 5’ adaptor ligation, and library amplification. To minimize bias,

various techniques are attempted, such as modified two-adaptor ligation, circularization, the introduction of unique molecular identifiers, polyadenylation, and the use of template-switching oligos (30). While some poly(A)-tailed asRNAs and eRNAs have been documented, most lack a poly(A) tail and are not easily detected by conventional RNA-seq. Therefore, proper sequencing-based methods for the identification of asRNAs and eRNA involve profiling nascent or newly transcribed RNA by either biochemical enrichment or the use of modified nucleotides. Various strategies allow for nascent RNA detection, including STARR-seq, caRNA-seq, NET-seq, PRO-seq and TT-seq (31). Although each of these sequencing methods has strengths and limitations, the nuclear run-on-based sequencing method, PRO-seq provides information about each ncRNA's expression level and length together with coding gene transcription. Notably, compared to the other ncRNAs, the role of eRNAs in immune cells, especially Th cells, has not been rigorously studied (**Fig. 1D**). Recently, eRNAs have emerged as potential diagnostic markers and even therapeutic targets due to their involvement in diverse gene expression mechanisms, including enhancer-promoter looping, chromatin modification, phase separation, and regulation of transcriptional machinery (32). Future studies of the functional details of eRNAs in Th cells are required to gain a better understanding of Th cell function and fate decisions.

3D chromatin organization

Cell differentiation and function of Th subsets require precise control of gene expression. 3D genome organization, which largely relies on architectural proteins such as CCCTC-binding factor (CTCF), cohesin, and YY1, has emerged as another layer of gene expression control involving CREs. The architectural proteins facilitate short-range chromatin contacts arising from E-P interactions as well as megabase-scale structures demarcated by boundaries known as TADs. By controlling E-P interaction specificity, 3D chromatin architecture is a crucial feature of the spatiotemporal regulation of gene expression (33). Our understanding of 3D genome organization has dramatically improved during the past decade by virtue of a series of chromosome conformation capture (3C)-based techniques coupled with NGS technology. 3C-based technologies using chemical cross-linking followed by enzymatic digestion of chromosomes and proximity ligation have enabled high-throughput and genome-wide detection of contact frequencies between genomic loci (34). Among the 3C-based methods, 4C-seq and HiC (or its derivatives, including PLAC-seq and micro-C) are used to investigate chromatin contacts on a scale of one locus to all loci and all loci to all loci, respectively (**Fig. 1E**). Although genomic interactions of several individual loci have been reported in Th cells using 3C or imaging methods (35,36), only a few open-ended genome organization studies using 4C-seq and HiC have been conducted (37-39). Thus, future work will need to provide insights into the impact of genome structure on characteristic traits of Th cells.

EPIGENETIC REGULATION OF Th PROGRAMS

Epigenetic mechanisms control gene expression at each stage of Th cell differentiation beginning with lineage-defining *Cd4* expression. Expression of the hypermethylated *Cd4* locus is activated by TET1/3-mediated DNA demethylation with the aid of multiple CREs. Whereas a CRE located 14kb upstream of the *Cd4* transcription start site (TSS) (E4P) dictates *Cd4* expression initially in thymocytes, a 2kb downstream intronic CRE (E4M) modulates *Cd4* expression during lineage commitment and maturation of CD4+ T cells in the thymus (40). Peripherally, epigenetic regulatory machinery inhibits premature cytokine expression in naive T cells that have not yet polarized to a particular subset. Mechanistically, this

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involves the accumulation of repressive H3K27me3 marks at loci like *Ifng* and *Il4* (41,42). In addition, the *Il4* and *Ifng* loci of naive T cells maintain DNA methylation that is absent in cytokine-positive cells (43,44). A similar repressive H3K27me3 pattern is evident among TFs linked with differentiation, including *Tbx21* (Th1), *Gata3* (Th2), and *Rorc* (Th17), thereby inhibiting differentiation programs that are silenced in the absence of antigenic stimulation (41). Thus, multiple epigenetic components contribute to the suppression of effector programs in naive T cells.

Upon activation, CD4+ T lymphocytes undergo epigenetic reprogramming, which includes the deposition of histone acetylation and other permissive histone marks (H3K4me3), leading to characteristic gene expression changes. Accordingly, transcriptomic and epigenetic profiling can discern the emergence of distinct CD4+ T cell lineage subsets (45). In particular, the histone acetyltransferase enzyme p300 is enriched at super-enhancers of cytokines and cytokine receptors, including *Ifng*, *Il10*, *Il17a*, *Il17f*, *Il2ra*, and *Il7r*; however, it is most prominently enriched at the *Bach2* locus, which encodes a key negative regulator of effector T cell differentiation (42,46,47). The STAT4-T-bet axis is responsible for histone acetylation at the *Ifng* gene in Th1 cells, while the STAT6-GATA3 axis is responsible for histone acetylation at the *Il4* locus in Th2 cells (42). At the *Il4* locus, T-bet-mediated reduction of histone acetylation is observed, whereas GATA3 achieves a similar effect at the *Ifng* gene (42). In addition, it was recently shown that the Th17 master transcriptional regulator retinoic acid-related orphan receptor γt (RORγt) is required to induce chromatin accessibility of IL-17A, IL-17F, and IL-23R via recruitment of the SWI/SNF chromatin remodeling complex (48). Indeed, the landscape of CREs and their associated chromatin architecture varies remarkably among polarized Th cells. The epigenetic mechanisms underlying naïve T cell polarization to each Th lineage, especially the activation of master TF, are reviewed below. Notably, the various master TFs themselves are often epigenetically regulated and usually impact the epigenetic status of target loci upon binding their cognate sites.

Th1 lineage

Th1 cells are characterized by the secretion of IFN-γ and play essential roles in the immune response to intracellular microbes. Signaling pathways triggered by IFN-γ and IL-12 activate STAT1 and STAT4, respectively, which induce the master TF of Th1, T-bet. In collaboration with Hlx, Runx3, and Ets family members, T-bet transcriptionally activates *IFN-γ* and simultaneously represses *IL4* expression (49). Along with STAT4-dependent activation of the Th1-specific enhancer landscape (50), T-bet activates most of the Th1 cell-specific genes, including cytokines, chemokines, and chemokine receptors, that are required for the function of Th1 cells (51). The expression of the *Tbx21* gene, encoding T-bet, is controlled by multiple TF-bound CREs. TCR and co-stimulatory cytokine signals induce STAT1, NFAT, AP1 and NF-κB binding at Tbx21 promoter to elicit *Tbx21* transcription (52), while a CRE located 12kb upstream (CNS12) of *Tbx21* mediates IL-12-induced T-bet expression in Th1 cells by recruiting STAT1 and STAT4 and is also responsive to IFN-γ stimulation (53) (**Fig. 2A**).

T-bet can modulate Th1-specific gene expression via epigenetic mechanisms involving PTMs on histones and chromatin organization. T-bet interacts with histone demethylase Jmjd3 to induce Th1 genes by removal of repressive H3K27me3 marks (54). Alternatively, T-bet may directly promote H3K27me3 deposition a subset of Th2 signature genes, including *Gata3*, to inhibit their expression in Th1 cells (55). IL-2-inducible T cell kinase-mediated phosphorylation of T-bet promotes its interaction with GATA3, thereby sequestering the master TF factor of Th2 cells from its target sites and further enforcing the Th1 phenotype

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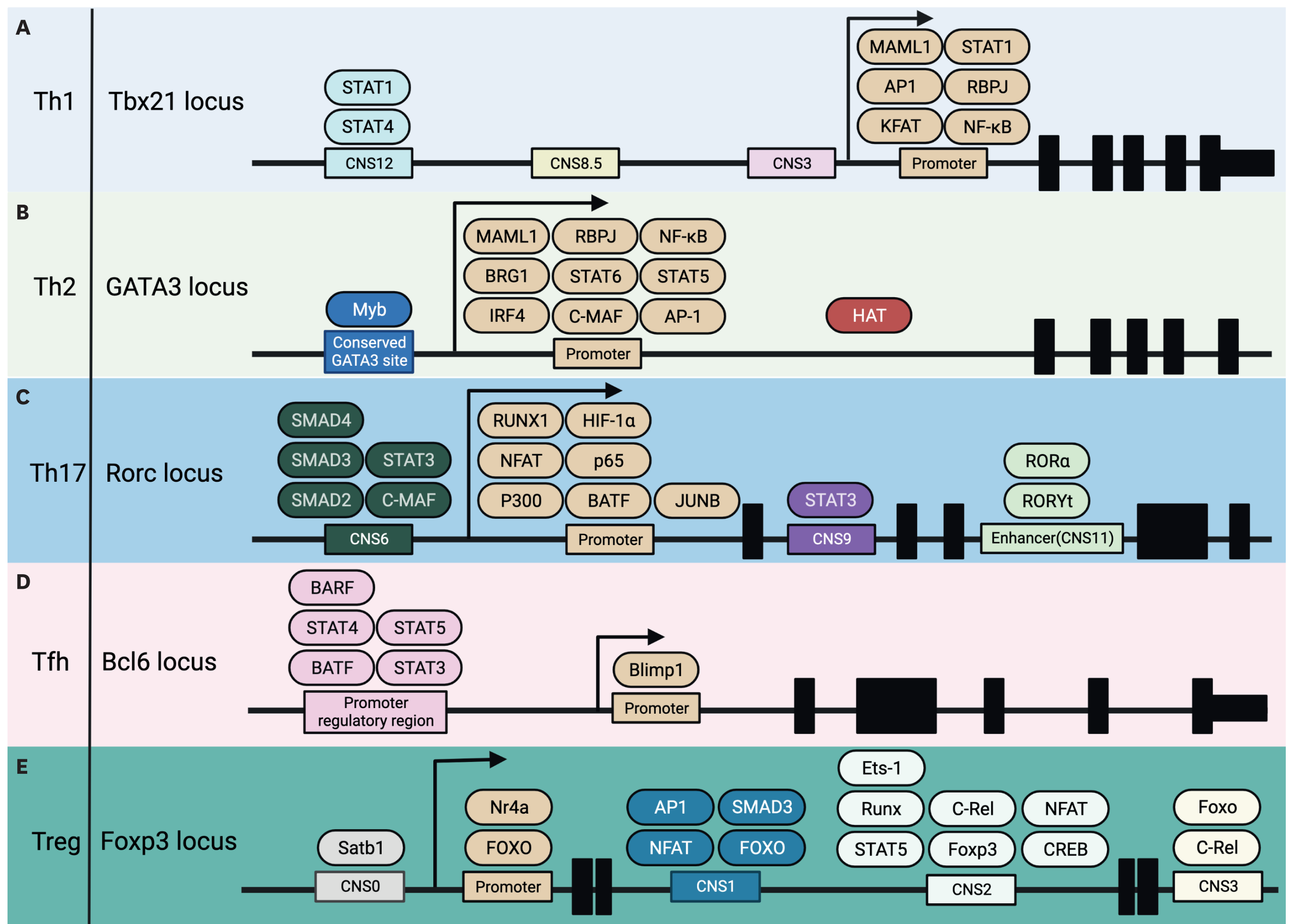

Figure 2. Schematic diagram of Th cell lineage-determining TF loci. (A) The STAT binding motif in CNS12 of the *Tbx21* locus is important for IL12-induced *T-bet* expression during Th1 cell differentiation. The *Tbx21* promoter recruits various TFs to facilitate transcription in Th1 cells. (B) The repressive PcG complex negatively regulates the *GATA3* locus but is displaced from the promoter upon IL4-mediated STAT6 activation and promoter binding, which allows for Th2 cell differentiation. A HAT complex also contributes to activation via permissive histone acetylation. (C) CNS6 and CNS9 of the *Rorc* locus are important for Th17 cell differentiation. IL-6 signaling prompts STAT3 to bind CNS6 and CNS9 for *RORγt* induction, while CNS6 also mediates TGF-β-dependent *RORγt* expression. (D) The *Bcl6* locus contains a promoter regulatory region located at -11 kb that recruits several TFs to activate *Bcl6* transcription. (E) *Foxp3* is regulated by IL2. CNS0, CNS1, and CNS3 recruit multiple TFs to induce *Foxp3* while CNS2 maintains *Foxp3* expression.

(19). Direct binding of T-bet to CREs at the *Ifng* locus, including multiple enhancers and the *Ifng* promoter, organizes a Th1-specific 3D genome architecture by enhancing occupancy of CTCF and cohesin (56). Although several studies have shed light on the CREs at key Th1 genes, including *Tbx21* and *Ifng*, additional genome-wide profiling of T-bet and other TF binding as well as epigenomic marks in combination with functional validation, using techniques such as CUT&RUN, (multi-) CUT&Tag (24), and CRISPR-mediated interference (CRISPRi) (8), is necessary to gain a better understanding of Th1 gene regulation.

Th2 lineage

Th2 cells express GATA3, secrete IL-4, IL-5, and IL-13, and can drive allergic inflammation. In response to IL-4, phosphorylated STAT6 induces a set of genes in naive CD4+ T cells that includes *Gata3*, the Th2 lineage-determining TF. Epithelial-derived cytokines thymic stromal lymphopoietin, IL-25, and IL-33 also play critical roles in the priming of Th2 cells (57). Th2 differentiation and phenotypic stability require robust GATA3 expression that involves

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multiple mechanistic features (**Fig. 2B**). In Th2 cells, *Gata3* is primarily regulated STAT6, which dimerizes upon phosphorylation and directly binds to *Gata3* CREs, including the proximal promoter, an intragenic region, and a distal promoter positioned 10kb upstream of the TSS (58). STAT6 binding causes the dissociation of Polycomb group (PcG) proteins, which mediate H3K27 methylation-dependent silencing, and the spread of Trithorax group (TrxG) proteins that are responsible for the deposition of permissive H3K4 methylation (59). The replacement of PcG complex proteins with those of TrxG at the *Gata3* locus ensures high expression of *Gata3* that is essential for Th2 polarization.

Genes of the characteristic Th2 cytokines IL-4, IL-5, and IL-13 are clustered in the Th2 cytokine locus (160kb in humans, 120kb in mice), and their transcription is controlled by multiple distal CREs comprising a locus control region (LCR), a conserved GATA response element, and other DHSs (59). As a pioneer factor with chromatin-remodeling capabilities, GATA3 binds to most of the CREs in the Th2 cytokine locus and is joined by other TFs, such as STAT5, STAT6, NFAT, and YY1 (60). 3C analysis of this locus showed spatial proximity not only between the *Il4*, *Il5*, and *Il13* promoters but also with distal CREs in a SATB1-, YY1-, and GATA3-dependent manner (35). In addition, Hi-C data indicated that GATA3 is associated with CTCF-mediated regulatory chromatin interactions on a genome-wide scale in Th2 cells (37). Alternatively, by interacting with repressor proteins, such as BCL11b and NuRD complex components, GATA3 can silence non-Th2 gene expression (61). This functional change largely depends on PTM of GATA3. For example, phosphorylation at Ser308, Thr315, and Ser316 by activated Akt serine/threonine kinase releases the histone deacetylase Hdac2 from a GATA3/CHD4 complex to de-repress *Tbx21* expression. Also, Arg261 methylation blocks IL-5 transactivation by promoting interaction of GATA3 with Hsp60 and preventing its association with AP-1 and ETS1 (62,63).

Given the data available data on long-range interactions in Th2 cells, including possible LCR inter-chromosomal associations (64), further mechanistic studies are needed to appreciate the Th2-specific interplay between enhancers and promoters as well as its impact on gene expression. In addition, it should be feasible to identify more disease-relevant regulatory elements of key Th2 genes, using CRISPR-powered fine mapping that is informed by GWAS data and potentially complemented by eQTL analyses as well as epigenomic profiling, as recently demonstrated for *GATA3* (65).

Th17 lineage

Th17 cells, distinguished by their production of IL-17A, IL-17F, IL-21, and IL22, are essential for mucosal immunity against extracellular pathogens but also promote autoimmunity and chronic inflammation. RORγt and a (RORa) are the master TFs for Th17 differentiation and function (66). The expression of *Rorc*, which encodes RORγt, is initiated through the activation of an alternative promoter (RORC2) that contains NFAT and NF-κB binding sites. At least two cytokines, IL-6 and TGF-β, are required for *Rorc* up-regulation and the initiation of Th17 cell differentiation (67). Based on alignment of the human, mouse, dog, and rat genomic sequences at the *Rorc* locus, there are 11 conserved regions that are putative enhancers. Among those conserved non-coding sequences (CNSs), CNS6 and CNS9 recruit regulatory TFs and are required for the deposition of permissive epigenomic marks, including H3K27Ac and H3K4me3 at the *Rorc* locus. Furthermore, genetic disruption of CNS6 or CNS9 decreases Th17 cell differentiation and protects against Th17-mediated autoimmune disease *in vivo*. Both CNS6 and CNS9 contribute to IL-6 induced *Rorc* expression through STAT3 binding with or without SMADs, respectively, while CNS6 is also indispensable for TGF-β-induced *Rorc* expression via STAT3 binding (68). The *Rorc* enhancer RORCE2, which

overlaps CNS1 and CNS2, is required for proper differentiation of Th17 cells through its SOX5 and STAT3-dependent looping with the *Rorc* promoter (36). An intragenic enhancer located at +11kb of *Rorc* (CNS11) that cooperatively binds RORα and RORγt is crucial for the maintenance of *Rorc* expression and Th17 phenotypic stability (17) (**Fig. 2C**).

RORγt cooperates with multiple TFs, including STAT3, IRF4, BATF, RORα and RUNX, to transcriptionally regulate *IL17a* and other Th17 genes. These TF interactions and RORγt turnover are tightly regulated via ubiquitination, sumoylation, phosphorylation, and acetylation (69). RORγt also recruits histone-modifying enzymes, such as p300 and JMJD3, to promote permissive epigenomic changes and chromatin remodeling (69). Although mechanistic studies have been conducted for several example loci, like that of *IL17a,* the genome-wide resolution of RORγt binding and its interplay with epigenomic marks is still not sufficient to explain transcriptional regulation in Th17 cells, due in part to limitations of the ChIP-seq method. Future studies, employing more sensitive ChIP-seq refinements, such as ChIP-exo, or possible alternatives, like CUT&RUN and CUT&Tag, will need to confirm and extend previous results to achieve a clearer mechanistic understanding of Th17 gene expression.

Follicular helper T (Tfh) lineage

CXCR5-expressing Tfh cells are critical for mediating humoral immune responses against pathogens by assisting B cells in their maturation and differentiation. Commitment and maintenance of the Tfh lineage are promoted by autocrine signaling of IL-21 (70). B-cell lymphoma 6 (BCL6) is the lineage determining TF for Tfh differentiation and function (71), and its expression is regulated by complex signaling circuits involving multiple TFs (reviewed in (72)). *In vivo,* IL-6 is a key signal that induces *Bcl6* in CD4+ T cells, via STAT1 and STAT3 promoter recruitment, to initiate the Tfh differentiation program (73). The Th1 cytokine IL-12 also induces the expression of *Bcl6* by suppression of H3K27me3 that is dependent on phospho-STAT1 and STAT4 binding (74). Whereas Batf is a positive regulator of *Bcl6* that directly binds to the promoter and upstream enhancers (75) (**Fig. 2D**), FOXO1 negatively regulates *Bcl6* expression upon binding to a region near the TSS (76).

BCL6 protein can function as an obligate transcriptional repressor by interaction with multiple corepressors, such as BCOR, NCOR1, and NCOR2, Accordingly, it regulates Tfh down-regulated genes and up-regulated genes by at least two modes of action, namely direct repression and repression-of-repressor mechanisms, respectively (75). For example, BCL6 suppresses lineage-determining TFs, including *Tbx21*, *Gata3*, *Rora*, and *Rorc*, via direct binding and upregulates the Tfh-specific gene *CXCR5* by countering the repressor Blimp-1 (77). During Ab affinity maturation in the germinal center, B cells undergo significant changes in 3D genome structure, including at the *Bcl6* locus, to facilitate lineage-specific gene expression (78). However, it is currently unclear if similar alterations in chromosome topology occur during Tfh differentiation.

Treg lineage

The Treg subset of CD4+ Th cells is specialized in suppression of pathological immune reactions against self and non-self Ags. Tregs developed in the thymus in response to self-Ags presented by thymic Ag-presenting cells, such as thymic dendritic cells and thymic epithelial cells, or can differentiate in the periphery upon exposure to specific environmental cues (i.e., commensal microbiota or food Ags) in the context of TGF-β stimulation. Foxp3 is the lineage determining TF for Tregs, and its expression, which is sufficient to confer an immune-suppressive phenotype in T cells, is tightly controlled.

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The *Foxp3* locus harbors at least four distinct CNSs, based on comparative genomic analysis, that feature binding sites for various TFs and function as enhancers (79) (**Fig. 2E**). While the *Foxp3* promoter has a basal transcriptional activity that is up-regulated by binding of Foxo1, Foxo3, Nr4a, NFAT, or other TFs. The lineage-specific expression of *Foxp3* is largely driven by the enhancer elements (80). Two intragenic enhancers, CNS1 and CNS2, play a role in extrathymic Treg differentiation and stable *Foxp3* expression in response to TCR stimulation, respectively. CNS2, in particular, has a high CpG content, and CpG demethylation, mediated by the TET2 enzyme, is essential for its enhancer function (81). CNS3, which is located near the transcription termination site, and upstream distal enhancer CNS0 are indispensable for thymic Treg generation. H3K27ac HiChIP demonstrated that activated CNS0 and CNS3 engage in enhancer-enhancer and enhancer-promoter looping at the *Foxp3* locus beginning in the early stages of Treg cell development (82).

Various molecular mechanisms, including an array of PTMs and protein-protein interactions, underlie Foxp3-dependent transcriptional regulation of Treg differentiation and phenotypic stability. PTMs, including phosphorylation, ubiquitination, acetylation, and methylation, can modulate the activity and stability of Foxp3. More than 15 residues of Foxp3 are known to be phosphorylated by multiple kinases. The kinases either augment (NLK) or suppress (CDK2, PIM1, and PIM2) Foxp3 function. Foxp3 protein levels are also controlled by reversible acetylation via the actions of histone acetyltransferases (HATs) and histone deacetylases (HDACs). In some cases, modifying enzymes compete for the same residue, as can occur at lysine residues targeted for mutually exclusive acetylation and ubiquitination, or are otherwise impacted by nearby modifications. Thus, there is potential for substantial cross-talk between distinct modifications, much of which remains to be fully resolved (83). The mechanistic details by which PTMs fine-tune FoxP3 transcriptional activity have been reviewed elsewhere (84).

Foxp3 can interact with multiple key TFs and co-factors (85) with context- and partner-dependent effects (85). For example, Foxp3-Runx1 and Foxp3-RORγt complexes suppress Th1 genes (IL2 and IFN-γ) or Th17 genes (IL-17A), respectively. Meanwhile, a Foxp3-IRF4 complex activates genes involved in Th2 suppression and a Foxp3-GATA3 complex activates the genes encoding each of its components (86). Structural analysis revealed that Foxp3 can form a domain-swapped homodimer that positions two distinct FoxP DNA-binding sites in close proximity (87). This bridging potential of Foxp3 suggests that it can control Treg-specifc gene expression by re-organizing the 3D genomic structure (87). Consistently, 4C-seq anchored at the Foxp3-bound *Ptpn22* locus demonstrated that Foxp3 can modulate the Treg chromosomal 'interactome' (38), while a global assessment, based on Foxp3 HiChiP, showed enrichment of Foxp3 association with promoter-enhancer loops at loci it regulates (87).

Additional genome-wide studies should help to address many unresolved mechanistic questions regarding Foxp3 function. For example, it is still debated whether FoxP3 is a transcriptional activator, a repressor, or both (88). It is also unclear whether the up- or down-regulated genes in differentiated Tregs are directly or indirectly regulated by Foxp3. Unlike other lineage-determining TFs, Foxp3 does not seem to induce extensive chromatin remodeling, as most Foxp3 binding sites are already accessible across many different immune cell types from the stem cell stage (89). Accordingly, Foxp3 likely engages in alternative epigenetic strategies to control target gene expression in Tregs that remain to be uncovered.

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EPIGENETIC CONTROL OF Th LINEAGE PLASTICITY

Although Th cell lineages can be discretely categorized based on their sensitivity towards different pathogens, hallmark cytokines, master transcription factors, and specific gene expression programs, the existence of CD4+ Th cells with ambiguous classification and their potential contributions to disease trajectories is now appreciated. These hybrid Th lineage subsets have been reported both *in vitro* and *in vivo* (90-93).

Th cell plasticity is contingent on the co-expression of lineage-determining TFs (94). T-bet and GATA3 co-expressing Th1/Th2 hybrid cells can be induced *in vitro* by activating naïve CD4+ T cells with IFNγ, IL-12, and IL-4 and are observed *in vivo* in intestinal helminth infections (91). Tregs exhibit highly flexible master TF expression patterns. Foxp3+ Tregs co-expressing various lineage-determining TFs, including T-bet, GATA3, and RORγt, have been reported and the presence of these additional master TFs can enhance Treg suppressive function in multiple disease contexts (94). The Tfh cell lineage also displays substantial heterogeneity. Depending on the cytokine milieu, Tfh1 producing IFNγ and IL-21, Tfh2 producing IL-4 and IL-21, Tfh17 producing IL-17 and IL-21, and T follicular regulatory cells can be generated. These hybrid subsets co-express the lineage-determining TFs BCL6 and T-bet, GATA3, RORγt, or Foxp3, respectively (70).

Similarly, multiple studies have documented extensive Th17 cell plasticity. The Th1 transcription factors STAT4 and T-bet, upon activation by IL-12 and IL-23, can convert Th17 progenitors into Th1-like cells (95). Consistently, in IL-17F reporter mice with colitis, Th17 cells converted into IFN-γ-producing cells that were associated with disease progression (95). Th cells co-expressing a second lineage-specific transcription factor, such as GATA3 or Foxp3, in Th17 cells along with RORγt have been identified (96). These double-positive cell populations possess functional characteristics and can express cytokines of both subsets (93,97). For example, GATA3+ RORγt+ T cells can produce IL-17 and IL-4, the characteristic cytokines secreted by Th17 and Th2 subsets, respectively. Th cells co-expressing RORγt and Foxp3 can be detected in the mouse intestine without any treatment and have enhanced immunosuppressive activity relative to Foxp3+ cells in the context of intestinal inflammation (97). Moreover, these RORγt+Foxp3+ Th cells show minimal CpG methylation at key Treg loci, including *Foxp3*, suggesting phenotypic stability conferred by epigenetic changes (96). A genome-wide DNA methylation analysis on *ex vivo*-expanded CD4 T cells revealed a methylation signature in the Th17 subset that is more similar to that of naïve CD4 T cells than Th1 cells. In fact, the most demethylated areas were detected in Th17 cells, which is consistent with their observed plasticity (96). Locus-specific DNA methylation analysis of Th17 cells versus non-classic Th1 cells expressing both IFN-γ and IL-17 also provided evidence for Th17 plasticity, showing that promoters of *RORC2* and *Il-17* are demethylated in non-classic but not classic Th1 cells (98). In addition, the Th1 cell-like phenotype induced by the treatment of Th17 cells with IL-12 *in vitro* is associated with a decrease in permissive H3K4me as well as histone acetylation at the Il-17 locus and an increase in these modifications at the *Ifng* locus (99). Thus, although more genome-wide data are needed, epigenetic alterations that occur concomitant to and are likely consequent of expression of an additional master TF seem to stabilize Th cell plasticity, and Th17 cells may be particularly amenable to the acquisition of alternative phenotypes as a result of their epigenetic landscape.

These findings also indicate that Th differentiation programs are not mutually exclusive even though each Th cell subtype has multiple mechanisms to enforce its selected lineage

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and inhibit other possible identities. Th cells co-expressing lineage-specific master TFs add diversity to context-specific immune responses and are functionally important. Notably, global mapping of H3K4me3 and H3K27me3 marks in CD4 T cells revealed the unanticipated presence of both on the promoters of lineage-specific transcription factors (93,95,97,99-102). This bivalency at master TF loci may largely explain the potential of CD4 T cells for phenotypic conversion. Intriguingly, this contrasts with the pattern of histone modifications that prevails at various cytokine loci in each CD4 T cell lineage, which is characterized by permissive marks decorating hallmark cytokine loci and repressive chromatin at cytokine genes associated with alternative lineages (41,49). For instance, *Il17* and *Il21* loci are marked by repressive H3K27me3 in Th1, Th2, and regulatory T cells but feature permissive H3K4me3 in Th17. These results support prior *in vitro* research indicating that signature cytokines of a particular Th subset are highly repressed in other subtypes but also underscore the regulatory complexity of CD4+ T cell lineages and their potential for interconversion (41,49).

DISEASE ASSOCIATION OF Th LINEAGE PLASTICITY

Cancer

Epigenetic regulation of Th lineage plasticity in the tumor immune microenvironment (TIME) may affect the prognosis of cancer patients. Since Th functional plasticity is associated with epigenetic changes, epigenomic analysis can be used to characterize immune cells that accumulate in the TIME. The DNA methylation landscape of tumor-infiltrating CD4+ T cells from glioblastoma patients exhibits distinct patterns relative to blood CD4+ T cells. In the former, methylation correlates with transcriptome alterations for 341 genes. Thus, the TIME may trigger epigenetic modifications that facilitate gene expression changes in infiltrating CD4+ T cells (103). The H3K27me3 methyltransferase EZH2 is a potential therapeutic target in many cancers. Tregs show distinct expression levels of EZH2 depending on their location. Those present in the tumor are characterized by high levels of EZH2 and H3K27me3 compared with Tregs found in non-lymphoid tissues, resulting in tumor tolerance (104). Similarly, in melanoma patients, EZH2 expression is elevated in tumor-infiltrating versus peripheral blood Tregs. In addition, EZH2 and H3K27me3 levels are specifically increased in Tregs compared to other CD4+Foxp3- T cells in tumor tissues. Inhibition or genetic ablation of Ezh2 in the Tregs of tumor-bearing mice decreases FOXP3 expression and alters their immunosuppressive function. Indeed, Ezh2-deficient, tumor-infiltrating Tregs up-regulate pro-inflammatory cytokines, including TNF-a, IFN-g, and IL-2, and down-regulate IL-10. This phenotype is associated with increased recruitment and function of CD8+ and CD4+ effector T lymphocytes in the TIME, promoting tumor eradication in mice (104). Thus, targeting EZH2 in Tregs can remodel the TIME to enhance the antitumor immune response and should be validated in additional cancer models.

The presence of Th17 cells in the TIME is correlated with poor prognosis in colorectal cancer (CRC) patients (105). Th17 recruitment to the TIME may be mediated by interaction with CCL20, which is up-regulated by the lncRNA u50535 that promotes tumor growth and metastasis in CRC and has been shown to promote Th17 accumulation in cervical cancer (106,107). Epigenetic manipulation of Th17 cells in the TIME has not been explored and therefore its therapeutic potential is unclear but could have applications in multiple cancer types.

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Chronic inflammation

Th17 plasticity has been implicated in chronic inflammation, including inflammatory autoimmune diseases. Two pathogenic Th cell types, exTh17 and exTreg, are found in patients with chronic inflammatory conditions, and their abundance is associated with disease induction and progression. exTh17 cells, which not only secrete IL-17 but also have an IFN-γ-producing Th1-like phenotype, infiltrate sites of inflammation in both intestinal bowel disease (IBD) and rheumatoid arthritis (RA) (108,109). IL-12 seems to play a pivotal role in exTh17 differentiation, and immunopathology coincides with the appearance of these Th17-derived IFN-γ producers. In the context of intestinal inflammation caused by *Helicobacter hepaticus*–induced typhlocolitis, IL-17A+IFN-γ+ cells display increased permissive H3K4me3 and reduced repressive H3K27me3 marks at the *Il12rb2* locus, suggesting the expression of functional IL-12R (110). In *ex vivo* and *in vitro* generated Th17 cells, IL-12 signaling reduces H3K27me3 at the *Tbx21* locus, resulting in increased T-bet expression. These epigenetic changes trigger the differentiation of exTh17 cells by concomitant silencing of the *Rorc* gene in a STAT4/T-bet-dependent manner (111). exTh17 cells are thought to derive from the Th17 subset, given that they maintain expression of the Th17 lineage maker CD161 (112). Depletion of the zinc finger protein Casz1, which functions to limit the deposition of repressive histone modifications in favor of permissive marks at specific Th17 loci, including *Rorc* and *Il17a*, causes a significant reduction in IFN-γ+IL-17A+ inflammatory Th17 cells in mucosal Candida infection (113). Despite the lack of Casz1 DNA-binding data, this result is consistent with a Th17 derivation of IFN-γ+IL-17A+ cells and indicate that an epigenetic mechanism is at least partially involved. Interestingly, deficiency of T-bet not only leads to a marked absence of Th1-like IFNγ-expressing exTh17 cells but also blocks the generation of IL-17/IFN-γ double-producing cells that arise initially (114).

The number of IL-17A-producing Treg cells, or exTregs, increases in the intestinal mucosa and circulation of IBD patients as well as the inflamed joints of RA patients (108,109). The stepwise increase in the quantity of RORγt+ Tregs expressing IL-17, IFN-γ and TNF from IBD to early dysplasia and ultimately CRC suggests that their involvement in immune dysregulation fosters cancer-promoting chronic inflammation (115). Epigenetic and transcriptional studies have uncovered mechanisms by which Treg-specific β-catenin activation promotes the disease-associated RORγt+ Treg phenotype. DNA demethylation of *Foxp3* promoter CpGs, which allows access to TFs for stable gene expression, is a distinguishing feature of Tregs (116), and, as expected, Foxp3 expression seems to be directly involved in the emergence of exTregs. In collagen-induced arthritis, a mouse model of RA, TNF receptor 2 (TNFR2) signaling prevents polarization of Tregs toward a Th17 phenotype by maintaining CpG methylation at the *Foxp3* locus (117). Notably, CD4+ T cells from RA patients exhibit reduced Foxp3 levels. This may be attributable, at least in part, to decreased levels of the H3K4 methyltransferase ASH1L, which normally facilitates Treg differentiation indirectly by countering HDAC1 repression of Smad3-mediated *Foxp3* expression (118).

In general, Th17 cells are important contributors to the early phase of inflammation, and Th17 subsets, including hybrid lineages, facilitate chronic inflammation in the perpetuation of inflammatory diseases (109). Additional insights, provided by epigenomic interrogation and perturbation studies, are necessary to understand the mechanistic basis of Th17 and Treg plasticity in the context of inflammatory pathology and to inform the design of novel therapeutic interventions.

EPIGENETIC DRUG TREATMENTS AND THEIR IMPLICATIONS FOR T CELLS

Epigenetic marks can be modulated as a viable treatment strategy for various pathological conditions, including hematologic cancers and chronic inflammatory diseases in which T cells have a primary or important contributing role. Moreover, agents that elicit epigenetic changes can be harnessed to alter Th cell polarization and impact their plasticity in diverse clinical contexts. In theory, epigenetic drugs can be designed to target modified chromatin substrates (histones or DNA), their binding proteins (readers), or the modifying enzymes (writers and erasers). Therapeutic interventions employing epigenetic drugs have advanced considerably in the past couple of decades with dramatically improved target specificity and pharmacokinetics, although achieving cell type-specific effects remains an ongoing challenge. In particular, the advent of CRISPR-based strategies has engendered much anticipation for the clinical availability of treatments involving epigenetic manipulation of a single locus or even multiple, dispersed loci associated with disease (8).

Targeting DNA modifications

The first epigenetic drug to advance to clinical application was the nucleoside analog 5-azacytidine (5-azaC), which initially entered clinical trials as an anticancer agent for the treatment of acute myelogenous leukemia (AML) in 1967, over a decade prior to the emergence of key insights into its potential mechanism of action (119). This compound differs from cytidine by the presence of a nitrogen atom instead of carbon at the C5 position of the aromatic ring. The related drug 5-aza-2′-deoxycytidine (5-aza-dC), or decitabine, is structurally identical except for the absence of a 2’-OH in the ribose moiety. Following the stepwise addition of a triphosphate group, and removal of the 2’-OH in the case of 5-azaC, the resulting nucleotide analogs can be incorporated into DNA by polymerases. DNMTs, which target the C5 position of cytosine to yield the prevalent modified base 5mC, are effectively choked by these analogs, resulting in covalently trapped DNA adducts that are subsequently degraded. At high concentrations, 5-azaC has pronounced cytotoxic effects, which was the original impetus for testing it as a leukemia treatment, but this feature also limited its clinical utility initially. However, it functions effectively as a hypomethylating agent (HMA) when administered at lower doses.

DNMTs tend to favor cytosine modification at CpG dinucleotides, which are underrepresented in the human genome but tend to be enriched in relatively high GC content-containing regions, or CpG islands (CGIs), that are associated with many gene promoters (120). CGI hypomethylation is conducive to proximal transcriptional activity, and, conversely, high 5mC content of CGI promoters is repressive, as documented for various tumor suppressor genes, which can be de-repressed in oncogenic cells by extended exposure to low-dose 5-azaC or 5-aza-dC. Reduced methylation of hypermethylated gene bodies, which is a common feature of oncogenes and contributes to their over-expression, also has been demonstrated for 5-aza-dC (121). In addition, cancer-specific changes in methylation patterns on enhancers and super-enhancers, as revealed by genome-wide analysis (122), are likely impacted by these drugs and contribute to their effects on gene expression. Recurrent mutations in modifiers of DNA methylation, including DNMT3A and the dioxygenase TET2 that generates the 5mC oxidized derivative 5-hydroxymethylcytosine (5hmC), as well as factors that indirectly affect their activity, such as isocitrate dehydrogenase 1 and 2 (IDH1/2), are commonly observed in myeloid malignancies (105) and can be predictive of responsivity to HMA-based treatment (123). Both drugs have gained approval by the United States (US)

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Food and Drug Administration (FDA) for the treatment of bone marrow abnormalities in myelodysplastic syndromes (MDSs), and 5-azaC also has been approved for elderly patients with AML, for which MDS is predisposing, while 5-aza-dC is used off-label for this indication in the US (124).

Notably, the clinical effects and efficacies of the two compounds are not identical, likely because the ribonucleoside 5-azaC is primarily incorporated into RNA. Moreover, it is now apparent that multiple mechanisms, in addition to epigenetic changes in DNA methylation patterns, may account for their anticancer properties (124). A recently discovered non-nucleoside small-molecule inhibitor of DNMT1, GSK3685032, which was shown to be more efficacious and better tolerated in a mouse model of AML than 5-aza-dC possibly as a result of its improved specificity, has yet to enter clinical trials (125).

Both direct and indirect effects of HMAs on T cell function in cancer have been documented. In multiple cancer types, 5-azaC has been shown to increase tumor immunogenicity by up-regulating tumor-associated Ags to promote T-cell infiltration (126). Alternatively, the drug can de-repress endogenous retroviruses to induce an interferon response that sensitizes oncogenic cells and tumor tissue to T cell-mediated immune checkpoint therapy (124,127). In peripheral T-cell lymphomas (PTCLs), which involve neoplastic mature T or natural killer cells and tend to be aggressive, treatment with 5-azaC can elicit positive outcomes and durable responses; however, the mechanism of action, whether epigenetic or otherwise, has yet to be determined (128). HMAs may also have applications involving T cells that extend beyond cancer therapy. In cultured T cells, 5-azaC suppressed activation, proliferation, and pro-inflammatory cytokine production upon stimulation. Extended 5-azaC treatment resulted in the demethylation of the *Foxp3* promoter and concomitant up-regulation of *Foxp3*, leading to the expansion of Tregs. *In vivo*, 5-azaC conferred protection in a mouse model of graft-versus-host disease, suggesting its potential utility as an immunomodulatory agent in tissue transplantation (129).

Targeting histone modifications

Acetylation

The first epigenetic drugs targeting histone modifications to reach the clinic were HDAC inhibitors (HDACis), with suberoylanilide hydroxamic acid (SAHA), or vorinostat, receiving FDA approval in 2006. SAHA was approved for the treatment of cutaneous T cell lymphomas (CTCLs), a group of rare cancers initially presenting as skin lesions. A second HDACi, romidepsin, gained FDA approval as a therapy for CTCL in 2011, and, a few years later, for PTCL. SAHA potently inhibits all 11 human HDACs, whereas romidepsin is a selective agent with potent activity against class I HDACs, which includes HDAC 1, 2, 3 and 8. Remarkably, these initial HDACis have had only a narrow scope of application in the clinic so far, and, even when deployed against the aforementioned T cell-derived cancers, they are not frontline treatments. A second generation of HDACis with improved pharmacokinetics have been developed, including the SAHA-derivative belinostat that also was approved for the treatment of PTCL by the FDA in 2014 (119). While available clinical data generally indicate a lack of efficacy for HDACis when administered alone for other cancers, a broader potential in oncology might be realized in combination with HMAs, chemotherapy, immunotherapy, or other modalities (119,130). T cell exhaustion is one of the major problems in immunotherapy and functional restoration may be possible with HDACis. In mouse models, HDACi treatment augments the immune response of exhausted T cells upon viral infection, and a combined immunotherapy of anti-PD-1 with an HDACi conferred improved survival to melanoma (131,132).

It is unclear why T cell lymphomas are uniquely sensitive to this class of epigenetic drugs as single agents, but enhanced responses also might be achieved with multimodal regimens (130). Furthermore, possible applications of HDACis unrelated to cancer therapy with direct or indirect effects on T cells have emerged and are currently being evaluated in clinical trials. These include treatment of HIV-1 patients with a first- (SAHA and romidepsin) or second-generation (panobinostat and chidamide) HDACi to reactivate latent virus in CD4+ T-cells, which can serve as a reservoir that prevents complete viral elimination in the context of standard combined antiretroviral therapy (cART), offering the possibility of an elusive cure (119). Indeed, the establishment and maintenance of latency, by integrative viruses like HIV-1 as well as other non-integrative viral pathogens, is under epigenetic control and, in cell and animal models, usually vulnerable to HDACi treatment (133). However, in HDACi-treated, HIV-positive individuals on a cART regimen, even when viral reactivation is achieved, effective elimination of the virus has not been demonstrated, suggesting the need for alternative combinatorial treatments (134). In addition, clinical trials testing ameliorative effects of SAHA in neurodegenerative diseases, including Huntington's disease and amyotrophic lateral sclerosis (ALS), are currently underway (119). Neuroinflammation is now appreciated as an important pathological feature of these diseases, and pronounced central nervous system infiltration of activated CD4+ and CD8+ T lymphocytes has been demonstrated for ALS in particular (135); however, the extent to which HDACi efficacy is due to cell- autonomous vs non-cell autonomous effects and whether it is linked to changes in T-cell epigenetic profiles remains unclear.

Small-molecule inhibitors of bromodomain-containing proteins, which function as readers of N_{ε}-acetylated lysine residues, also have been investigated extensively due to their potential clinical utility. In particular, drugs targeting bromo- and extra-terminal domain (BET) proteins, including BRD2-4 and BRDT, have received considerable attention; however, numerous clinical trials evaluating their anti-cancer activity as monotherapies or in combination treatments have yet to yield any approvals, largely due to excessive toxicity, which may be attributable to insufficient specificity among BET family members for some compounds, or a lack of response in patients (136,137). Nevertheless, given that BET inhibitors have anti-inflammatory properties by virtue of their direct effects on various immune cells, including multiple T cell subtypes, BET inhibitors may have additional applications. Possible T cell-related indications include suppression of Th17 differentiation in autoimmunity (138) and conditions with a strong inflammatory component, including type 2 diabetes (136), as well as reactivation of latent HIV-1 in CD4+ T cells likely in combination with other modalities (134).

Methylation

Epigenetic drugs directed against histone methylation writers and erasers are also being investigated but are mostly in earlier stages of development (119). Lysine and arginine methyltransferases use the metabolic cofactor S-adenosylmethionine (SAM) as a methyl donor, and drugs with distinct mechanisms of inhibition, including competitive SAM-mimetics, SAM-uncompetitive inhibitors, and allosteric regulators have been described (119,139). Several compounds have advanced to clinical trials for the treatment of various cancers, including B-cell lymphomas. A small collection of cancer-related clinical trials are also underway for multiple lysine demethylase (KDM) inhibitors with activity against the enzyme LSD1, while inhibitors for other KDMs as well as arginine demethylases are much less developed. Collectively, only limited results from clinical trials seeking to modulate histone methylation are currently available (119), indicating varied success, and potential applications of this class of drugs to T cell-associated pathology, cancer or otherwise, remain undefined.

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Other histone marks

The epigenetic landscape is the composite of an expansive catalog of histone post-translation modifications, only a few of which have been targeted effectively in the clinic. Beyond acetylation and methylation, substantial progress has been made in modulating the nucleic acid modification poly(ADP-ribose) (PAR) for therapeutic purposes. PAR is the enzymatic product of poly(ADP-ribose) polymerases (PARPs) and is derived from nicotinamide adenine dinucleotide. PARylated chromatin, mediated by PARP1 and PARP2, is a key feature of the DNA damage response but also has been implicated in transcriptional regulation of gene expression. In both scenarios, PAR may have similar effects as an epigenetic mark, contributing to DNA accessibility, nucleosome remodeling and/or re-positioning, short and long-range chromosomal interactions as well as transient recruitment of various chromatin-associated proteins (140). Olaparib, which shows a preference for PARP1 over PARP2 but has nanomolar inhibitory activity against both, was the first PARP inhibitor to achieve FDA approval in 2014 for the treatment of ovarian cancer in patients harboring mutations in the homology-directed repair (HDR) proteins BRCA1 or 2. Subsequently, FDA approval has been extended to multiple PARP inhibitors for the treatment of breast, prostate, and a few other cancers with specific mutational characteristics, usually involving compromised HDR but in some cases independent of BRCA1/2 status (141). Notably, more Tregs are present in PARP1 knockout mice relative to wild-type littermates in lymphatic organs (142). Moreover, PARP inhibition enhances Treg suppressive activity (143), which has been attributed to a de-stabilization of Foxp3 by covalent PARylation. Collectively, these observations suggest that Treg number and suppressive function can be modulated and could have implications for how PARP inhibitors are deployed in cancer treatment while possibly justifying their broader application to autoimmune or chronic inflammatory diseases (143). Importantly, the effects of PARP inhibitors on genome-wide PAR distribution have not been profiled in any disease or treatment context, and thus the true epigenetic impact of these drugs, versus simultaneous non-chromatin alterations, and its relationship to efficacy will require rigorous investigation.

Cellular therapeutics and site-specific epigenetic treatments

Like other types of blood cells, autologous and allogenic T cells can be manipulated and expanded *ex vivo* prior to adoptive transfer into patients. In cancer immunotherapy, harvested tumor-infiltrating lymphocytes and CAR T cells designed to recognize and target tumor-specific Ags have been employed for adoptive cell therapy (ACT). It is now apparent that $CD4^+$ T cells, long known to infiltrate tumors alongside effector $CD8^+$ T cells, can be actively involved in tumor eradication, contingent on their polarization (144). Moreover, the long-term persistence of activated $CD4^+$ CAR T cells as recently demonstrated in two former chronic lymphocytic leukemia patients more than a decade following ACT, maybe a key determinant in the durability of effective treatments (145). Interestingly, CAR T cells could have clinical utility beyond cancer therapy, targeting fibrosis in the heart or other tissues for example, and may be amenable to *in vivo* production via targeted, lipid nanoparticle-encapsulated mRNA (146). While the presence of Tregs in tumors is generally counterproductive in anti-cancer therapies, there is accumulating evidence, gleaned from numerous clinical trials, that these immunosuppressive cells can be harnessed for the treatment of various autoimmune conditions, such as type 1 diabetes, neurodegenerative disease, and in tissue transplantation. Notably, as Treg phenotypic stability is under epigenetic control, treatments involving one or multiple drugs targeting epigenetic regulators have been tested pre-clinically to maintain their functional polarization (147).

The introduction of CRISPR-based technologies has revolutionized precision medicine and affords the promise of truly targeted epigenetic therapies. The prokaryotic type II CRISPR/

CRISPR-associated protein 9 (CRISPR/Cas9) system, which functions as a primitive immune strategy, was first appropriated for sequence-specific genome editing. Subsequently, mutation of the Cas9 endonuclease yielded an enzymatically-dead version that retained RNA-guided localization (148) and, when fused to a chromatin-modifying effector protein or domain (148,149), allowed for selective epigenome editing. Deactivated Cas9 (dCas9) has been outfitted with both repressive and activating features to achieve CRISPRi or activation (CRISPRa), respectively. These effector functions have undergone multiple iterations and extensive optimization to date to enhance robustness and allow for broader applicability (8). Chromatin marks that have been effectively targeted include DNA methylation (5-mC and 5-hmC), histone acetylation (H3K27Ac), and histone methylation (H3K9me and H3K27me). In addition to altering chromatin modifications, dCas9 fusions also have been employed to modulate other epigenetic features, such as ncRNA expression/localization, genome architecture, and the epitranscriptomic mark m^6A in mRNA or ncRNAs. Multiple clinical trials utilizing active Cas9 to introduce genetic modifications in blood cells *ex vivo* prior to infusion and even a few involving *in vivo* genome editing are underway. A clinical trial employing a dCas9-linked base editor is also ongoing. Thus, while testing of CRISPR-based epigenetic treatments is still pre-clinical, there is much anticipation for this alternative approach with broad potential, which has additional appeal regarding safety in not being associated with an inherent risk of creating permanent, albeit rare, off-target genetic mutations. Indeed, it is possible to envision the future application of CRISPR-based epigenetic tools as an *ex vivo* and *in vivo* strategy to manipulate T cell plasticity to treat or prevent disease.

CONCLUSION

During the last decade, tremendous progress has been made in our understanding of Th cells with regard to epigenetic mechanisms and the epigenomic landscape by using advanced sequencing technologies. Probing of chromatin accessibility, TF binding, histone and DNA modifications, and contact frequencies of the 3D genome structure have allowed deeper insights into the regulation of gene expression in immune cells, including Th subsets. Master TFs are crucial for the regulation of each lineage-specific gene program and thus the establishment and maintenance of Th cell identity. Accordingly, their expression is tightly regulated by multiple epigenetic mechanisms, including chromatin remodeling, histone modification, and genome organization. However, it is now apparent that Th cells have the capacity for substantial phenotypic flexibility among these lineages, which is also under epigenetic control. This plasticity may be beneficial for host defense but also can contribute to pathology in certain disease contexts. Moreover, Th phenotypic plasticity may be amenable to manipulation with epigenetic drugs or tools in therapeutic interventions. Despite considerable advances in elucidating the molecular mechanisms underlying the differentiation, maintenance, plasticity, and function of Th cells, further studies are necessary to address many unresolved questions. For example, at the *Tbx21* and *Bcl6* loci, comprehensive identification and functional validation of CREs is still needed, comparable to the Foxp3 locus. The 3D genomic features of key Th gene loci also must be assessed with unbiased methods. In addition, the epigenomic landscape of mixed phenotype Th cells should be investigated systematically. Indeed, additional insights will supplement our current understanding to promote the development of novel strategies for treating immune-related pathologies, including cancer and chronic inflammatory diseases.

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