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Gene Regulatory Circuits in Innate and Adaptive Immune Cells

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Abstract

Cell identity and function largely rely on the programming of transcriptomes during development and differentiation. Signature gene expression programs are orchestrated by regulatory circuits consisting of *cis*-acting promoters and enhancers, which respond to a plethora of cues via the action of transcription factors. In turn, transcription factors direct epigenetic modifications to revise chromatin landscapes, and drive contacts between distal promoter-enhancer combinations. In immune cells, regulatory circuits for effector genes are especially complex and flexible, utilizing distinct sets of transcription factors and enhancers, depending on the cues each cell type receives during an infection, after sensing cellular damage, or upon encountering a tumor. Here, we review major players in the coordination of gene regulatory programs within innate and adaptive immune cells, as well as integrative omics approaches that can be leveraged to decipher their underlying circuitry.

INTRODUCTION

The human body and its composite cells have been called living machines since the dawn of medicine. Like nearly any machine, at least those that run on electricity, each cell type relies on a complex circuitry, most of which is devoted to the control of gene expression. The switching on or off of these circuits begins at the earliest stages of development. In pluripotent cells, a small node of circuits controlled by factors like NANOG, SOX2, and OCT4 becomes active, but the vast majority of other gene expression circuits remain poised. As embryonic development proceeds, many of the poised circuits are shut off in each cell type, coinciding with a successive loss of pluripotency. However, each cell type, in turn, activates new circuit modules that are regulated by so-called master transcription factors (TFs) that cement their identities. These TFs control expression by binding *cis*-regulatory elements (CREs) in the genome, such as promoters or enhancers, the latter usually located within intronic or more distal noncoding regions. A similar scenario plays out during immune cell lineage decisions and during functional differentiation of mature myeloid and lymphoid cells. In this review, we focus on regulatory circuits and TF programs that govern entire modules of gene expression in innate and adaptive immunity, endowing cell identity and modulating the functional state of a given cell.

GENE REGULATORY CIRCUITS

Herein, we consider a regulatory circuit to be composed of the TFs, *cis*-acting elements, and requisite chromatin changes that coordinate the expression of a gene in a given cell type under certain conditions, or states. As such, the circuitry for a single gene is not always the same. A gene circuit can be rewired, perhaps using different sets of enhancers and TFs in different cell types or when the same cell type encounters distinct microenvironmental cues. One can appreciate the complexity of underlying regulatory logic for these circuits given the need to control expression of ~25,000 human genes in more than 200 cell types using the ~400,000 putative enhancers cataloged to date (1, 2). Indeed, genes whose activities are controlled in time-, location-, and stimulus-specific manners are often linked to multiple, even dozens, of enhancers, each contributing to the overall specificity of gene expression (3).

Gene promoters and enhancers, as well as other flavors of CREs, serve as conduits for TFs that, in turn, recruit other nuclear proteins to revise local epigenetic landscapes. These proteins write or erase DNA and histone modifications, alter chromatin accessibility, or stabilize interactions with the transcriptional machinery. The first identified epigenetic modification in mammals was DNA methylation at CpG dinucleotides, which forms 5meCpG (4, 5). These dinucleotides are enriched at promoters, where CpG islands enforce transcriptional silencing when methylated or activation when demethylated. In addition to this simple binary code, dozens of distinct modifications can be placed on the N-terminal tails of histones 3 and 4 (H3 and H4), forming a complex alphabet that is read to facilitate or repress transcription (6–8). The two most commonly studied histone modifications for transcriptional regulation are acetylation and methylation (mono-, di-, or trimethylation) of H3/4 lysine residues (**Figure 1*a*,*b***).

Like any language, the histone code is established by (*a*) writers, enzymes that add chemical groups (e.g., histone acetyl transferases and histone methyltransferases); (*b*) erasers, enzymes that remove chemical groups [e.g., histone deacetylases (HDACs) and histone demethylases]; and (*c*) readers, protein modules that bind a chromatin modification and translate it into a specific biological function (e.g., bromodomains and chromodomains for acetylation and methylation, respectively) (6). Each of these word processors is recruited to a given locus through TF-bound regulatory elements (REs) or by binding directly to a set of preprinted histone modifications. In brief, active genes and chromatin regions are typically enriched for acetylation marks, such as

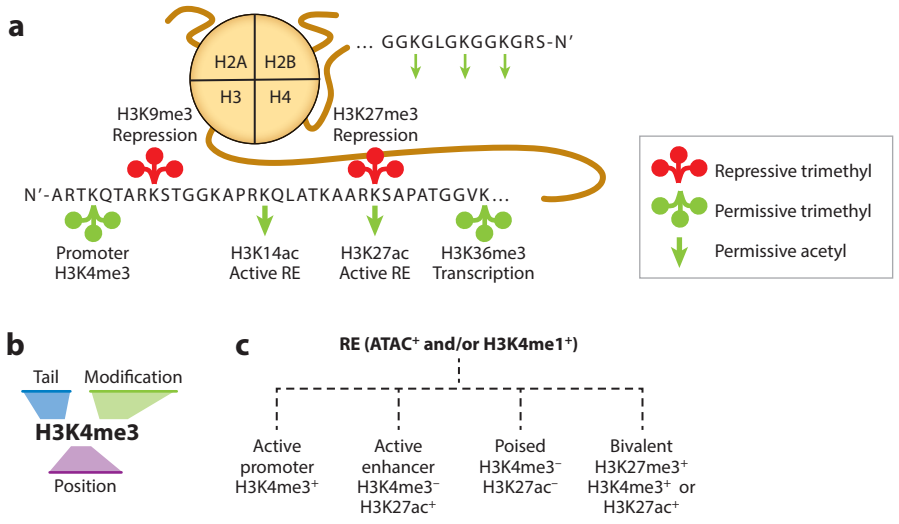


Figure 1

The histone code. (*a*) Highlighted are the histone tail modifications mentioned in the text. Those below the histone tail are activation-associated modifications (*green arrows* are acetylation and *circles* are methylation), and those above are generally repressive (*red circles*). For methylation, each circle represents one methyl group. (*b*) A key to the nomenclature and moieties. (*c*) Major modifications are shown that associate with different classes of accessible REs (i.e., ATAC$^+$). Abbreviations: ATAC, assay for transposase-accessible chromatin; RE, regulatory element.

H3K27ac, at active enhancers (9, 10), which are deposited by polymerase complex member P300 (11). For lysine methylation, H3K4me1, H3K4me3, and H3K36me3 are hallmarks of active/poised enhancers, active promoters, and transcription, respectively, whereas H3K27me3 and H3K9me2/3 are repressive marks associated with heterochromatin (10, 12–14) (**Figure 1*a–c***).

DECIPHERING GENE REGULATORY CIRCUITS AND PROGRAMS

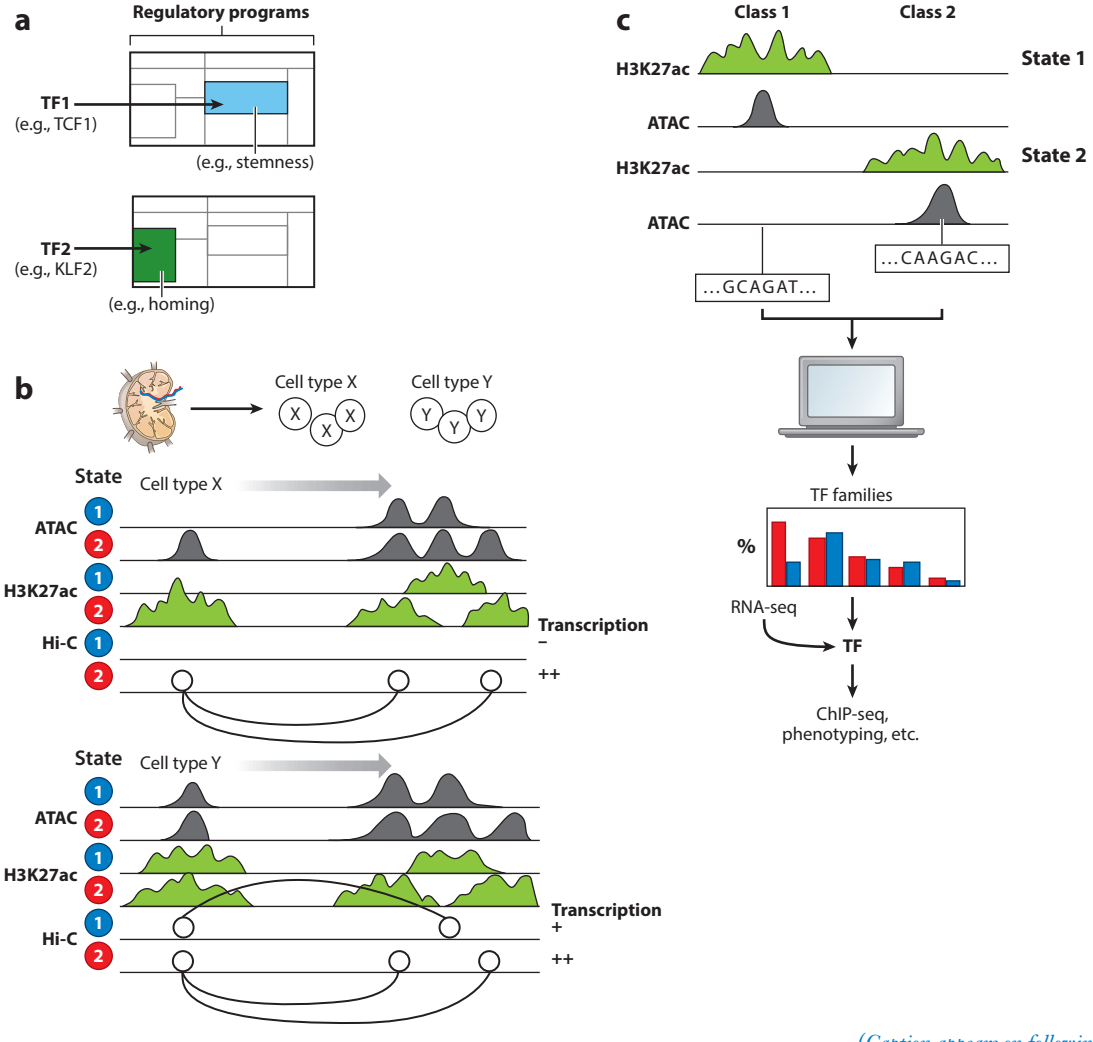
In the last decade, there have been major efforts to map the collection of CREs that participate in gene regulatory circuits, including initiatives by the ENCODE and IMMGEN consortia (15, 16). The emerging data have been especially valuable for deciphering another layer of biology—gene regulatory programs that guide immune cell development or their responses to a variety of stimuli. Herein, we refer to programs as the collection of regulatory circuits coordinated by key TFs that activate or inactivate cohorts of genes governing cell identity and function (also referred to as functional nodes or modules) (**Figure 2*a***).

A typical starting point for deciphering gene regulatory programs is to sort the cell type of interest and, under the desired conditions, characterize its transcriptome and map its open chromatin regions (**Figure 2*b***). The latter method tags genomic locations of poised or active REs with relatively high resolution. Traditionally, this has been accomplished using DNase-seq. However, the assay for transposase-accessible chromatin using sequencing (ATAC-seq) has emerged as the predominant method due to its ease of application in a range of cell types and its requirement for very low cell input (~10^4 for sorted populations or even a single cell). These data yield accessible RE maps but are not the optimal metric for relative RE activities, which are best monitored by chromatin immunoprecipitation sequencing (ChIP-seq) for specific histone modifications, especially H3K27ac for enhancers and H3K4me3 for promoters. Again, recent technical advancements

have enabled analysis of histone modifications on samples with limited cell input (routinely ~10^4), including CUT-and-RUN (cleavage under targets and release using nuclease) sequencing and ultralow input (ULI)-ChIP-seq (17–20).

Integration of chromatin accessibility and histone modification maps in a given cell state permit classification of REs as active, poised, or silent. Coupling the information with transcriptome data, one can then proceed to parse out active versus inactive gene regulatory circuits and the factors that may govern their status (15). For example, we and others have developed algorithms to identify enriched DNA motifs in classes of REs for a given cell type (e.g., all active enhancers) or when comparing two cell types [e.g., T helper type 1 (Th1) cells versus group 1 innate lymphoid cells (ILC1s)] (17, 18). This approach will reveal TFs that target enriched motifs to activate (or repress) whole cohorts of REs and, presumably, their gene targets (**Figure 2*c***).

Validation of a specific enhancer's importance in a given regulatory circuit can now be achieved via a number of CRISPR-based approaches, including enhancer deletions, mutations, repression



(*Caption appears on following page*)

Figure 2 (*Figure appears on preceding page*)

Deciphering gene regulatory circuits and programs. (*a*) Regulatory programs for a given cell state. Shown are examples of the gene expression modules (*blue* and *green*) governed by two distinct TFs, which are two components of the regulatory program for a given cell type and functional state. The underlying regulatory circuits for these expression programs are not shown. The examples are assigned as modules that mediate stemness (TCF1-based) and homing (KLF2-based) within naive lymphocytes. (*b*) Identification of regulatory circuits for a given gene (*gray arrow* indicating the direction of transcription) by chromatin profiling. Sorted cell types (X and Y) from a given sample (*top*) analyzed in two functional states (e.g., +/− agonist, normal versus disease, or two distinct tissues), as indicated by the numerals 1 and 2. Samples are initially subjected to chromatin profiling with ATAC-seq (accessibility) and ChIP-seq for H3K27ac (RE activity). Contacts between REs, including promoter-enhancer contacts, are determined by Hi-C or another conformation capture methodology. For cell type X, gene expression (transcription) in state 2 relies on a regulatory circuit consisting of the promoter (H3K27ac mark at 5′ end of the gene) contacting two active enhancers (two H3K27ac marks downstream of the gene). The middle enhancer is predicted to be irrelevant, converting from active to poised (state 1 to 2) with no promoter contacts in state 1. For cell type Y, distinct circuits are employed for either low-level (state 1; promoter–middle enhancer) or high expression (state 2; promoter–proximal + distal enhancers). (*c*) Workflow for motif analysis to identify important TFs. (*Top*) Enhancers are first classified based on activity profiles in distinct cell types or states. The DNA sequences corresponding to accessible regions (ATAC-seq peaks) are then extracted for each enhancer class. Sequence enrichment can be performed either de novo (i.e., any enriched sequence) or for known TF motifs (i.e., using a TF motif database). The resulting data set will predict TF families, rather than specific TFs, which can then be integrated with paired RNA-seq to predict specific TFs within a family. Predictions then can be validated by ChIP-seq or targeted perturbations. Abbreviations: ATAC-seq, assay for transposase-accessible chromatin sequencing; ChIP-seq, chromatin immunoprecipitation sequencing; Hi-C, high-resolution conformation capture; RE, regulatory element; TF, transcription factor.

(CRISPRi), or activation (CRISPRa). However, pairing enhancers with their target genes is not always straightforward, as most chromosomal neighborhoods have collections of genes interspersed with arrays of enhancers. In general, enhancers relevant to the circuitry of a given gene and cell state make physical contacts with their target promoters. These genomic contacts can be captured by chromatin cross-linking, using so-called C (conformation-capture) techniques, which now can be performed at very high resolution on a genome-wide (Hi-C) scale (21, 22). Thus, by integrating transcriptome, chromatin, and conformational data for a given cell type and state, one can develop testable hypotheses regarding the important REs and TFs governing a gene regulatory circuit, or nodes within the expression program, for that cell state. However, when testing such hypotheses, one must also be aware that most expression circuits have built-in redundancies, which may muddle interpretation of validation experiments or CRISPR-based screens over complex regulatory landscapes.

Notwithstanding, integrative chromatin approaches have produced impressive advances in our understanding of gene regulatory circuits and programs that govern the development (23–25), receptor repertoire formation (26, 27), and function of our immune system in both health and disease (28). We now describe a subset of such advances, with a focus on the regulatory circuits that drive biological processes and cell identities in both the innate and adaptive arms of our immune defenses.

REGULATORY CIRCUITS AND PROGRAMS IN HUMORAL IMMUNITY

Historically, the molecular programs of B cells and humoral immune responses have been some of the most intensively studied with regard to cellular differentiation, effector functions, and memory, and they will serve as an appropriate springboard for our discussion.

Mature B cells emerging in the adult bone marrow undergo extensive selection to prune out autoreactive clones and generate the peripheral repertoire. Upon antigen encounter, naive follicular B cells differentiate in the germinal center (GC), where they proliferate and execute programs for affinity maturation. Activated B lymphocytes, as well as those in the GC (GCB cells), can differentiate into plasma cells (PCs) and memory B cells, subsets that are responsible for antibody secretion and long-term protection, respectively. These cell fate decisions are

instructed mainly by the strength of signals received from B cell receptors (BCRs), pattern recognition receptors, and follicular T helper (Tfh) cells (CD40L and cytokines). Signal-responsive TFs then collaborate with other lineage-specific TFs to establish the regulatory programs for each cell fate.

In primary B lymphocytes, PAX5 and EBF1 are required for maintaining cellular identity (29). Master TFs also have been identified for the GCB and PC subsets, which include BCL6 and BLIMP1 (encoded by *PRDM1*), respectively. The memory B cell subset bears several striking resemblances to the naive B cell program, including expression of BACH2 and KLF2, both of which maintain a quiescent state and prevent premature differentiation into PCs via suppression of *PRDM1* (30, 31). Another TF, called HHEX, is critical for driving the GCB cell–memory B cell transition, where it is involved in a mutually repressive pathway with BCL6 (32).

One of the most extensively studied TF regulatory circuits in humoral immunity involves BLIMP1, IRF4, and BCL6, which largely govern GCB–PC programs. In naive or GCB cells, IRF4 is induced in proportion to signaling strengths from the BCR, and via CD40L on Tfh cells. A higher level of IRF4 (high-affinity BCR) induces expression of BLIMP1 and initiation of the PC program. In contrast, low to intermediate levels of IRF4 induce BCL6, which governs the GC program, including somatic mutation and selection to generate higher-affinity BCRs (33, 34). Both BLIMP1 and BCL6 are transcriptional repressors, directly inhibiting the expression of each other, which reinforces regulatory programs for PC and GCB cell fates, respectively (35, 36). In addition to this classical circuit, mathematical modeling unveiled the NF-κB subunit c-REL as a control for the switch between GCB proliferation and PC differentiation by antagonizing BLIMP1, which can otherwise directly repress c-REL expression (37).

As in most gene regulatory circuits, the collection of TFs that regulate B cell fate collaborate with epigenetic modifiers to revise chromatin and enhancer landscapes, generating cell type–specific transcriptomes. These relationships have been studied in detail using a T-independent activation model in which lipopolysaccharide (LPS)-stimulated B cells differentiate into PCs (38–40). While it is generally accepted that chromatin accessibility and H3K27ac densities positively correlate with RE activity, one critical challenge has been functional validation on a large scale. In this regard, a recent multi-omics study (3) identified the collection of active enhancers in LPS-stimulated B cells using a high-throughput screening method that assessed the transactivation potential of all accessible chromatin regions (41). Notably, active enhancer elements were often enriched for at least five to six TF motifs, compared with fewer in the inactive but accessible regions. Once the active RE cohort was defined, complementary Hi-C analysis revealed complex promoter-enhancer networks in the LPS-stimulated B cells, including promoters regulated by multiple enhancers (multi-enhancer genes), and enhancers that regulate multiple genes (multigenic enhancers). The latter were shown to coordinate metabolic and DNA or protein biogenesis pathways, whereas multi-enhancer genes are enriched in specialized pathways, including MHC-II presentation and the endoplasmic reticulum–associated degradation response. This study provides an exemplar for how integrative chromatin-transcriptome-conformational-functional analysis can lead to new biological insights and testable hypotheses.

In addition to chromatin accessibility, DNA methylation is an essential epigenetic mark controlling gene expression and cell differentiation. A systematic analysis of DNA methylomes during LPS-induced B cell differentiation to PCs revealed stepwise demethylation of CpGs in a broad collection of enhancers, which correlated with the extent of cell division (42). Motif analysis showed that early differentially methylated regions (DMRs) with hypomethylation were enriched in NF-κB and bZIP (AP-1) motifs, while late hypomethylated DMRs were enriched in IRF and POU (Oct-2) motifs, patterns that were largely recapitulated in analyses

of chromatin accessibility (43). Intriguingly, a majority of DMRs undergo hypomethylation and increased accessibility only in cells that have divided at least eight times (42, 43); many of these DMRs are associated with genes involved in mitosis and metabolism. These epigenetic processes potentially are mediated by TET (ten-eleven translocation) DNA 5-methylcytosine oxidases, which regulate B cell function and PC differentiation (44–46). Indeed, the link between cell division and demethylation is reminiscent of a recent finding that lymphocytes primarily demethylate DNA passively through TET-mediated DNA oxidation, which impedes CpG methylation during cell replication (47).

In vivo, it is clear that BCL6 serves as the master TF not only for GC reactions, governing the regulatory program of GCB cells, but also for the requisite T helper (Th) arm, Tfh cells (48–50), which rely on a similar BCL6-BLIMP1 repressor-of-repressor circuit (51). In Tfh cells, BCL6 also induces expression of chemokine receptors, such as CXCR5 and SIPR2, to promote B-Tfh retention and congregation at the GC (52). In addition, BCL6 regulates metabolic programs amenable to the GC microenvironment, including decreased glycolysis, increased lipid metabolism, and upregulation of the cytidine diphosphate–ethanolamine pathway (53–55). Similar to gene expression, several epigenetic regulators appear to operate in both GCB cells and Tfh. For instance, TET2 represses the expansion of both cell types, consistent with its role in suppressing GCB cell– and Tfh cell–derived lymphomas (45, 56). TET2 also facilitates differentiation of GCB cells to PCs (44, 45), while the de novo methyltransferases DNMT3A and DNMT3B oppose PC differentiation (57). Speaking to its universal role in lymphoid cells, BCL6 appears to govern regulatory modules in suppressive T follicular regulatory (Tfr) cells, which exhibit hallmarks of GC cells at both the chromatin and transcriptome levels (58).

During the dynamic transitions between GCB cell subsets, TF networks and epigenomes undergo substantial reorganization. Traditionally, GCB cells are divided into light zone (LZ) and dark zone (DZ) cells, and they continuously cycle between zones, where they undergo selection and massive proliferation, respectively. A recent multi-omics study suggested addition of a new GCB classification: gray zone (GZ) B cells, a subset of DZ B cells that are actively dividing (59). While previous analysis of LZ and DZ B cells showed remarkable similarity in steady-state transcriptomes (15, 60), the integrative epigenome-transcriptome approach revealed distinct signatures when the GCB classification was divided into LZ, DZ, and GZ. For instance, ATAC-seq analysis showed that DZ-specific regions are enriched for CTCF, FOXK1, and ZFP691 motifs; LZ regions are enriched in SPIB, PU.1, and IRF8 motifs; and GZ regions are enriched in OCT2, SP1, E2A, and EBF1 motifs. Thus, integration of multiple data platforms provides critical information about how TF networks collaborate to carve epigenomes and orchestrate the GC reaction for optimizing humoral responses.

TRAINED INNATE IMMUNITY

Protection from pathogens and malignancies requires a multilayered, cooperative network of innate and adaptive immune responses. Functionally, the innate immune system recognizes conserved structures on pathogens, which enables a prompt but nonspecific response to the invader. By comparison, adaptive immunity is slower in eliminating the primary infection but develops an efficient, clonally selected, and antigen-specific response capable of producing long-lasting immune memory (61). Historically, innate immune cells have been thought to lack memory programs, responding anew with each pathogenic insult. However, we now appreciate that innate immune cells exhibit memory-like characteristics that manifest as a state of high alert against reinfection, a process referred to as trained immunity or innate memory (62).

A majority of studies have focused on trained immunity in macrophage and natural killer (NK) lineages, but there is evidence for memory in other cell types, including ILCs and epithelial stem cells (63, 64). In contrast with adaptive memory, trained immunity predisposes innate cells for an enhanced secondary response against the same, or even a different, pathogen (64–67). Both microbial [e.g., bacillus Calmette-Guérin (BCG), *Candida albicans*, β-glucan, *Plasmodium falciparum*, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)] and nonmicrobial (e.g., Western diet, oxidized low-density lipoprotein, and uric acid) stimuli induce trained immunity in innate cells (67–71). Following SARS-CoV-2 infection, monocytes from convalescent individuals exhibit characteristics of trained immunity, with chromatin profiles implicating IRF1, SPIB, and NR4A1 as important components of the underlying regulatory program (71).

For macrophages, trained immunity has been studied most extensively using BCG vaccination or a β-glucan model. In primary or naive innate cells, antimicrobial response loci are mostly blanketed by repressive chromatin, including methylated CpGs, enforcing an off state for these circuits (72, 73). Upon initial exposure to BCG or β-glucan, proinflammatory loci are activated for expression (e.g., genes for TNF-α, IL-6, and IL-1β), remain epigenetically poised after removal of the stimulus, and are more rapidly transcribed upon a subsequent challenge. Primary exposures are accompanied by the revision of histone modifications at trained loci, including deposition of H3K27ac, H3K4me3, and H3K4me1, which support a stable open chromatin state capable of rapidly binding upstream TFs upon subsequent induction (67, 69, 74–78). Such epigenetic training is not restricted to mature effector cells but also can occur in developing progenitors. Upon vaccination and exposure to microbial ligands, hematopoietic stem cells (HSCs) remodel enhancer landscapes to generate licensed monocytes or macrophages that more potently respond to subsequent microbial challenges (79–81). While it is unlikely that a single key regulator exists for all trained immunity programs, important TFs have been described for select cell types. For example, polymorphisms in *HNF1a* and *HNF1b* correlate with BCG-induced training of human HSCs (81). C/EBPβ is another such factor that, when deleted in HSCs, results in a loss of LPS-induced open chromatin regions (82).

β-Glucan training of monocytes is a nice example of the interplay between cellular metabolism and epigenetic remodeling of gene regulatory circuits. Upon treatment with this fungal cell wall component, monocytes undergo a metabolic switch to aerobic glycolysis, which is dependent upon the dectin-1/Akt/mTOR/HIF-1α pathway (83). Tricarboxylic acid (TCA) cycle activity elevates fumarate levels in these trained monocytes, inhibiting histone demethylase KDM5 and then degradation of HIF-1α, which increases H3K4me3 and H3K27ac to augment the activities of select promoters and enhancers, respectively (84). In addition to metabolism, long noncoding RNAs (lncRNAs) also are predicted to play a role in β-glucan-induced trained immunity via epigenetic remodeling. Specifically, β-glucan induces an immune-priming lncRNA, UMLILO, which interacts with the WDR5-MLL1 complex, directing it to promoters of the innate response genes *IL6*, *IL8*, *CSF1*, and *CXCL*s, ultimately increasing H3K4me3 levels (76). Collectively, integrative chromatin/RNA profiling has provided new insights into how epigenetic reprogramming renders innate cells capable of rapid cytokine responses upon subsequent stimulation, a feature once regarded as unique to the adaptive immune system.

However, not all stimuli trigger a heightened inflammatory response upon a second encounter. For example, LPS treatment induces endotoxin tolerance in macrophages, which results in a lower inflammatory response upon subsequent exposure to this potent agonist (85, 86). Although the specific TFs governing LPS tolerance are still under investigation, this process is accompanied by a loss of active chromatin marks in monocytes at elements associated with key proinflammatory genes (74, 87, 88).

POLARIZATION OF ADAPTIVE AND INNATE LYMPHOCYTES

Upon encountering a pathogen, antigen-presenting cells (APCs) generate an effector response by presenting peptides to adaptive $CD8^+$ cytolytic T (Tc) and $CD4^+$ Th cells. The Th response develops over several days, during which time the cells proliferate and are directed toward a functionally polarized fate by cues from APCs, pathogen-associated molecular patterns (PAMPs), and other danger signals in their microenvironment. Each polarized Th subset expresses a signature cocktail of cytokines and other immune response genes tailored to eradicate certain types of pathogens. In general, there are three major effector immune responses (**Figure 3*a***). Type 1 immunity is generated when APCs recognize PAMPs common to viruses or intracellular pathogens, which triggers IL-12 and IL-18 production and, in turn, polarizes Th1 cells to produce IFN-γ. Type 2 responses are PAMP independent; instead, Th2 cells are produced upon recognition of signals common to tissue destruction in the wake of parasitic infections and allergens, which drive Th expression of IL-4, IL-5, and IL-13. Type 3 responses are initiated by fungal PAMPs, metabolic ligands, and APC-produced IL-23, which induce functional polarization to the Th17 fate and secretion of IL-17 and/or IL-22.

The stable clonal expression patterns of each polarized Th subset are enforced via epigenetic modifications within regulatory circuits for genes induced or repressed by the corresponding microenvironmental cues (89, 90). In all functional subsets, chromatin revisions are initiated by cytokine-STAT signaling: IL-12-STAT4 for Th1, IL-4/IL-5/IL-13-STAT6 for Th2, and IL-23-STAT3 for Th17. Once in the nucleus, the dimeric STAT factors recruit activating histone acetyl transferases and P300 to key enhancers that control specific functional modules in each Th subset (91). The STAT-dependent wave of chromatin revision allows expression of lineage-determining transcription factors (LDTFs) that further activate gene modules within their respective polarization programs.

Although originally attributed only to $CD4^+$ Th cells, polarization is now widely recognized as a biological process in other lymphoid lineages, including innate-like NK T (NKT) cells, γδ T cells, and ILCs. All of these subsets develop from common lymphoid progenitors but, unlike $CD4^+$ Th, NKT, and γδ T cells, the ILCs lack clonally distributed antigen receptors. Instead, ILCs respond rapidly to their local cytokine milieu, producing profiles of effector molecules that largely parallel their Th cell functional counterparts. Indeed, the development and responses of ILC1/Th1, ILC2/Th2, and ILC3/Th17 counterparts each depend on a similar set of master TFs. Here, we focus on concepts that unify or distinguish the various Th and ILC regulatory programs.

A core aspect of their polarization programs is that ILC-Th counterparts must activate unique gene modules, specific to a functional subset, while repressing modules for alternative fates. These regulatory strategies employ similar sets of TFs and chromatin modifiers but rely on distinct cues and kinetics. The master TFs that drive each ILC-Th fate have been known for some time, as follows: ILC1s, NKT1 cells, and Th1 cells require *Tbx21* (T-bet); ILC2s, NKT2 cells, and Th2 cells require GATA3; and ILC3s, NKT3 cells, and Th17 cells require RORC (reviewed in 92), with production of IL-22 in the latter dependent upon aryl hydrocarbon receptor (AHR) (**Figure 3*a***). Unlike ILCs, Th cells can differentiate into other functional subsets, including $Foxp3^+$ regulatory T cells (Tregs) and $Bcl6^+$ Tfh cells; Foxp3 and Bcl6 are TFs that do not appear to be expressed in any ILC subset (93, 94).

A second distinction between the ILC-Th counterparts is that epigenetic programs at key cytokine and effector loci are acquired early in ILC development. Thus, in the absence of infection, naive Th cells and mature ILC counterparts display significantly different chromatin landscapes at signature regulatory circuits (95). However, the ILC-Th circuitry and gene expression patterns converge upon responses to acute infection. The convergence of regulatory programs is

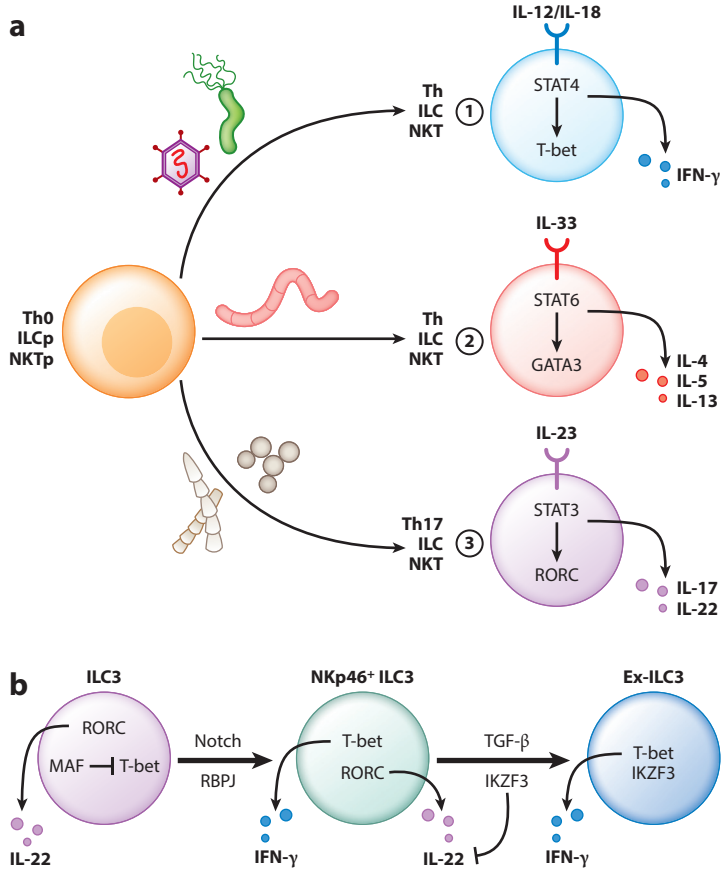


Figure 3

Regulatory programs for plasticity and functional polarization in helper lymphocytes. (*a*) Scheme for functional polarization within adaptive, innate, and innate-like helper lymphocytes. Upon activation, naive and progenitor lymphocytes (Th0, ILCp, or NKTp) will proceed along a polarization pathway based upon the type of infection and microenvironmental cues. Type 1, 2, and 3 immune cells are polarized in response to intracellular bacteria or viruses, helminths, and extracellular bacteria or fungi, respectively. Based upon danger signals and pathogen-associated molecular patterns, antigen-presenting cells release soluble cytokines (on top of cells) that activate JAK/STAT signaling through the relevant cytokine receptors. This is followed by activation of an LDTF by STAT, as shown, which drives initial stages of polarization. Signature transcription factors and cytokines for the three functional phenotypes are shown. (*b*) ILC3 plasticity. Indicated are cell types, transcription factors (*inside cells*), and soluble cytokines (*outside cells*) thought to be important for stepwise conversion of ILC3s to ILC1s. External signals that drive conversion events are indicated above the arrows. Abbreviations: ILC1, group 1 innate lymphoid cell; ILCp, ILC progenitor; LDTF, lineage-determining transcription factor; NKT, natural killer T; NKTp, NKT progenitor; Th, T helper; Th0, naive Th.

exemplified by elegant studies that examined accessible chromatin landscapes of ILC2s and Th2 cells in mice infected with a type 2 pathogen, *Nippostrongylus brasiliensis* (95). Specifically, Th2 cells developed chromatin accessibility at regions opened during ILC2 development, based on either unsupervised clustering or principal component analysis of ATAC-seq data. A similar convergence is observed in inflamed human mucosae, wherein signature cytokine loci exhibit nearly identical patterns of distal enhancer activity in Th1-ILC1 or Th17-ILC3 counterparts (18). At a molecular

level, motif imprinting predicts that the acquisition of shared regulatory programs is dependent upon LDTFs, such as GATA3, RORC, and T-bet, and TFs that activate numerous cytokine loci (e.g., *Runx1* and *Runx3*). Moreover, enhancers uniquely active in Th cells, not in ILCs, are enriched for motifs that bind TFs downstream of the T cell receptor (TCR), including the AP-1 factor FOS (18). Thus, while Th cells and ILCs share a core set of TFs, ILCs can activate a similar set of enhancers during their development, independent of TCR signaling. The precise mechanisms by which these regulatory programs are activated in ILC progenitors remain unknown.

Regulatory Programs for ILC-Th Plasticity

When examined ex vivo, functional polarization appears to be an irreversible fate decision coinciding with (*a*) a loss of chromatin bivalency (**Figure 1**), (*b*) acquisition of permissive chromatin at subset-specific regulatory circuits, and (*c*) deposition of repressive chromatin modifications at alternative fate loci, thus suppressing functional plasticity. However, certain innate and adaptive lymphoid cells retain at least a degree of functional plasticity in vivo. In these cases, the immature helper-type cell, upon receiving specific signals, can revise its chromatin landscape to that of another subset, conferring the immune system a degree of functional flexibility. Fate-mapping studies using LDTF reporter loci reveal conversion within some ILC or Th subsets, usually promoted by specific proinflammatory conditions. For example, single-cell chromatin profiling revealed that immature skin ILC2s retain a degree of accessibility at some enhancers containing RORC motifs, normally a characteristic of ILC3s (96). In an IL-23 injection model of psoriasis, these enhancers acquire additional accessibility, while other ILC2 enhancers lose accessibility. The converted ILC2s ultimately contribute to ~10% of the ILC3 population in psoriatic lesions (96).

Type 3 lymphoid cells also retain at least some degree of functional plasticity. As an example, in mice with sustained inflammation, most T-bet$^+$ Th1 cells were fate mapped as once being RORC$^+$ Th17 cells (97–100), indicative of a Th17-to-Th1 conversion. Similarly, ILC3s can produce a spectrum of T-bet$^+$ cells in response to their tissue microenvironments (101, 102), indicating a capacity for ILC3s to become ILC1s. One function of helper cell plasticity may be to control immunological tone within a tissue, which, when dysregulated, renders animals susceptible to disease and autoimmunity. For example, IFN-γ^+ ILC3s are enriched in the inflamed colon of Crohn disease patients (103, 104) and promote IFNγ-dependent colitis during *Campylobacter jejuni* infection or anti-CD40 administration (105, 106).

Epigenetic profiling has been an important approach for deciphering the precise mechanisms that govern Th17-to-Th1 and ILC3-to-ILC1 conversion (**Figure 3*b***). In one example, we used identification of superenhancers (17)—contiguous regions decorated by H3K27ac and harboring multiple powerful enhancer elements (107)—to find genes that help establish ILC and Th fates. Superenhancers associated with two genes, the surface receptor gene *CD300LF* and the TF gene *IKZF3* (encodes Aiolos), emerged as potential markers for cells transitioning from ILC3s (CD300LF$^+$Aiolos$^-$) to ILC1s (CD300LF$^-$Aiolos$^+$) (18, 108). In follow-up studies, we found that Aiolos binds putative *IL22* enhancers, reducing H3K27ac levels during the transition from ILC3 to ILC1 (108, 109).

Although sharing some TFs, the regulatory programs that govern functional plasticity in Th17 cells and ILC3s are characterized by distinct molecular targets. Both ILC3s and Th17 cells utilize RBPJ, a TF downstream of Notch signaling, and cMAF, as plasticity determinants. Within ILC3, cMAF and RBPJ suppress T-bet expression (110–113). In contrast, Th17 cells utilize these TFs to adopt an IL-10$^+$ regulatory phenotype (114, 115), which has not been identified reproducibly in ILC3s (93). One outstanding question that remains is why type 1 cells, unlike their type 2

and 3 counterparts, appear to lack functional plasticity. The key to resolving this question from mechanistic and biological standpoints will lie in more directed profiling of regulatory programs and their perturbation in Th1 cells, ILC1s, and NK cells.

Shared NK-CD8 Regulatory Programs

Similar to ILC-Th parallels, in many regards NK cells can be viewed as Tc cells that lack a TCR-dependent mode of activation. Upon initial encounter with antigen, Tc cells transition to memory and effector phenotypes, which ensures the generation of both self-renewing cells and highly cytolytic killers, respectively. A similar phenotypic divide occurs during the course of NK differentiation, in which these innate lymphocytes initially exhibit features common to naive or central memory $CD8^+$ cells (e.g., lymphatic homing and self-renewal) but also can differentiate into potent killers resembling effector Tc cells.

For $CD8^+$ cells, the naive stage is characterized by bivalent chromatin marks at promoters of effector genes, which are marked by both repressive (e.g., H3K27me3) and permissive (e.g., H3K4me3) histone modifications (116). Upon infection, these promoters lose their repressive marks, but nearby enhancers gain accessibility and H3K4me1/2, which allows epigenetic commitment to memory and effector fates (116). As expected, TCR and coreceptor stimulation leads to activation of enhancers bearing AP-1 and NFAT motifs (117, 118). A subset of TCR-mediated expression changes are epigenetically unstable, reverting upon antigen withdrawal. However, the stimulated $CD8^+$ cells stably activate a cohort of enhancers containing RUNX and T-box motifs, which drives gene modules for memory and effector functions (119). Importantly, RUNX3, along with T-box factors EOMES and T-bet, is critical for sculpting regulatory programs in both mature $CD8^+$ and NK cells, generating REs associated with memory loci that are both accessible and marked with H3K27ac (120–122).

Integrative chromatin-transcriptome analyses of human lymphoid cells revealed additional classes of enhancers in NK and $CD8^+$ T cell regulatory programs that distinguish self-renewing and dedicated effector cells (17, 123, 124) (**Figure 4**). In self-renewing $CD8^+$ cells (naive and central memory) or phenotypically equivalent NK cells ($CD27^+CD11b^-$ in mice and $CD56^{bright}$ in humans), TCF1, EOMES, ZEB1, and MYC are expressed, and target enhancers are enriched for their representative motifs (17, 111, 125, 126, 127). Within the NK cell lineage, TCF1 promotes the expression of cytokine receptor genes, including *Il7r*, and restrains effector functions, preventing NK cell maturation and granzyme expression (128). However, inactivation of the *TCF7* gene (encoding TCF1) in $CD8^+$ or NK cells produces relatively minor phenotypes during acute infection, likely because TCF1 has a close functional paralog called LEF1 that is expressed at lower levels (129). Notwithstanding, TCF1/LEF1 factors clearly play a pioneering role in activating REs that maintain self-renewal and helper phenotypes in $CD8^+$ and NK cells, but they may also contribute to repression of effector-linked REs via intrinsic HDAC activity (24, 130).

Upon activation and differentiation, effector $CD8^+$ and NK cells upregulate a new set of TFs, including T-bet, ZEB2, and BLIMP1 (131, 132). These components of the effector program cooperatively activate new genes and silence *TCF7* and its downstream self-renewal modules. The latter requires DNMT3-dependent methylation of *TCF7* and is accompanied by H3K9me3 chromatin deposition across *SELL* (encoding CD62L, which mediates lymphatic homing) and *IL7R* (mediates self-renewal) (133, 134). Accordingly, $CD8^+$ cells deficient in the histone methyltransferase SUV39H1, which imparts H3K9me3, fail to suppress memory programs (133). In addition to the CD8-NK axis, it is important to note that the TCF1-BLIMP1 counterbalance in programs associated with self-renewal versus quiescence is highly conserved through evolution (mouse to macaques to humans) and is applied in numerous lymphoid populations. The latter include Th1

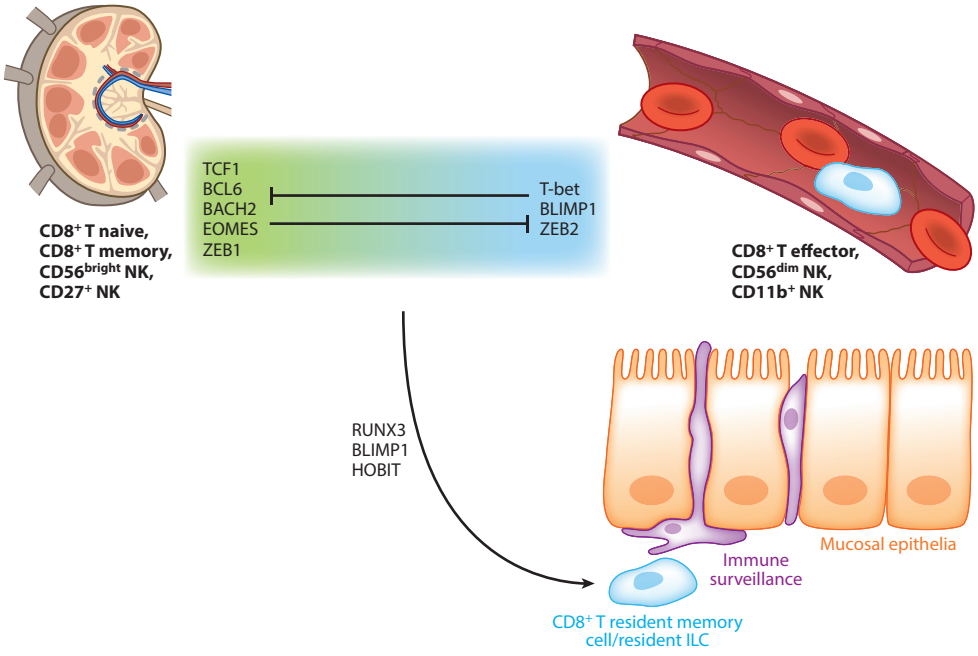


Figure 4

Regulatory programs conferring effector versus memory phenotypes, and tissue residency in NK and $CD8^+$ T cells. Diagram of tissue migration patterns and TFs shared by NK cells and $CD8^+$ T cells. (*Left*) $CD56^{bright}$ NK cells ($CD27^+$ NK cells in mice) and naive and memory $CD8^+$ T cells, which have a reduced potential for cytotoxicity compared to effectors. These cells home to the lymph nodes and express TCF1. (*Right*) $CD56^{dim}$ NK cells ($CD11b^+$ NK cells in mice) and effector $CD8^+$ T cells express high levels of cytotoxic effector molecules, are abundant in the circulation, and express the TF BLIMP1. Other TFs that mediate regulatory programs for cellular phenotypes, or mutually repress alternative phenotypes, are listed in the center. The blue-to-green gradient represents a transition in regulatory programs from one state to another. Select TFs contributing to tissue residency (e.g., mucosal lining) of memory $CD8^+$ T cells and ILC1-like NK cells are shown at the bottom. Abbreviations: ILC1, group 1 innate lymphoid cell; NK, natural killer; TF, transcription factor.

versus Tfh $CD4^+$ cells, memory versus exhausted cells in several lineages, and tissue-resident versus memory populations of lymphoid cells (135).

CD8-NK Memory and Tissue-Residency Programs

Conventional NK cells are short-lived terminal effectors, incapable of rapid expansion and, in several important ways, epigenetically distinct from $CD8^+$ cells. However, during murine cytomegalovirus (CMV) infection, adaptive-like NK cells bearing the Ly49H activation receptor are generated alongside their $CD8^+$ T cell counterparts (136). This NK population exhibits many CD8-like characteristics, including clonal-like expansion. In humans, CMV infection produces a similar NK subset, identified by upregulation of the NKG2C receptor (137), though it is unclear whether NKG2C participates as an activation receptor in recognition of human CMV–infected cells, akin to Ly49H recognition of murine CMV–infected cells in mice. Consistent with their phenotypic overlap, transcriptomes and regulomes of adaptive-like NK and $CD8^+$ effector cells are highly similar, in humans and mice (123).

Clonal expansion programs in $CD8^+$ and adaptive NK cells depend on signaling downstream of the proinflammatory cytokine IL-12, in which STAT4 serves as a pioneer factor to open chromatin at a shared cohort of REs (138), including those controlling additional TFs (139–141). The key TFs in this shared, adaptive NK-CD8 program have been identified as T-bet and EOMES, which function as master regulators of NK and $CD8^+$ cells; IRF8 and RUNX3, which control NK and $CD8^+$ cell proliferative bursts; and ZBTB32, which regulates proliferation and killing capacity (142–145). Only the latter TF appears to have some opposing roles in $CD8^+$ versus NK cells. ZBTB32 limits $CD8^+$, but not NK, cell expansion, possibly due to ZBTB32-mediated repression of BLIMP1 in NK cells (142, 146). Post-CMV infection, epigenetically altered NK cells return to a $TCF1^-$ basal state, unlike $CD8^+$ memory cells, which become long-lived $TCF1^+$ cells (140). Indeed, memory and memory-like populations produced from the two lymphoid lineages are clearly distinct, consistent with the observation that ILCs and T cells share some, but not all, functional circuitry.

Similar to effector and memory programs, chromatin landscapes of tissue-resident $CD8^+$ and NK cells are established by RUNX3, TCF1, and BLIMP1, or their paralogs (**Figure 4**). Upon exposure to TGF-β and IL-15, effectors in both lineages will convert into tissue-resident cells that are poorly cytotoxic but have a metabolic platform supporting robustness in mucosal or tissue microenvironments (147, 148). For NK cells, the converted populations are commonly called ILC1s, although a precise nomenclature remains controversial due to an absence of unambiguous NK versus ILC1 markers in humans (147). Active enhancers within ILC1s from oral mucosae are enriched for a cohort of TF motifs corresponding to SMADs, AP-1, and RUNX family members, while being depleted for BLIMP1 motifs (18). This TF profile likely corresponds to a generalized pathway in which strong TGF-β signaling triggers noncanonical SMADs to activate the appropriate adhesion genes. Conversely, repressors, such as BLIMP1 or its homolog HOBIT, silence REs that coordinate expression modules for circulation and cytotoxicity. However, one must be careful not to universalize these regulatory models given the wide range of variation when $CD8^+$ and NK cells reside in different tissues (149, 150). A possible exception to this cautionary note may be RUNX3, which has been shown critical for establishing chromatin accessibility in resident $CD8^+$ T cells across five tissues (151). These programs are also evident within tumor-associated $CD8^+$ cells, wherein RUNX3 controls the activation of residency-associated enhancer elements (151). However, tumor-resident Tc cells must contend with chronic stimulation, which engages additional regulatory programs that strike the delicate balance between effector functions and exhaustion.

Regulatory Programming of Exhaustion

After acute exposure, a subset of antigen-experienced $CD8^+$ T cells dedifferentiate into long-lived $TCF1^+$ memory cells (152, 153). However, during chronic viral infections or cancer, this pathway is perturbed and the cells enter an alternative state, often referred to as exhausted or dysfunctional. $CD8^+$ T cell exhaustion is characterized by a progressive decline in effector functions, reduced proliferation potential, and upregulation of inhibitory receptors, including PD1, TIM3, CTLA4, TIGIT, and LAG3. Integrated chromatin-transcriptome analysis revealed that exhausted $CD8^+$ T (Tex) cells have a landscape distinct from that of their effector and memory counterparts (154–160).

Acquisition of a fully exhausted phenotype is progressive, transitioning through several identifiable subpopulations, including (*a*) stem-like progenitors ($PD1^+Ly108^+TCF1^+$) that retain proliferation potential and effector functions, (*b*) intermediate Tex cells ($PD1^+CX3CR1^+T\text{-}bet^{hi}$) that are highly cytolytic, and (*c*) fully exhausted Tex cells ($PD1^+TIM3^+TCF1^-T\text{-}bet^{lo}$) characterized

by a complete loss of proliferative potential and effector cytokine production (161–163). Progression through this pathway is driven by underlying changes in TFs and epigenetic programs, which become less reversible, ultimately enforcing a heritable dysfunctional state in terminally differentiated Tex cells. Indeed, profiling of the open chromatin landscape within Tex subsets revealed a significant reduction of chromatin accessibility at genes regulating effector function and self-renewal capacity (e.g., *Ifng*, *Tbx21*, *Tcf7*, and *Lef1*) during progression to the fully exhausted state (161–163).

A recent study showed that de novo DNA methylation, mediated by the DNMT3A enzyme, is a critical component of the commitment to terminal T cell exhaustion (155). Accordingly, lymphocytic choriomeningitis virus (LCMV)-specific $CD8^+$ T cells from *Dnmt3a* conditional knockout mice fail to lose effector functions or proliferation capacity, despite persistent antigen stimulation during chronic infection. Instead, *Dnmt3a*-deficient $CD8^+$ T cells remain in a reprogrammable, TCF-1^+ stem-like state (155). This epigenetically reprogrammable state is of considerable therapeutic interest, since stem-like progenitor and transitory Tex cells are the main subsets that respond to immune checkpoint blockade used in cancer treatments. Thus, an in-depth understanding of molecular mechanisms that preserve functional plasticity in Tex cells, preventing their terminally exhausted fate, may enhance current checkpoint regimens or the efficacy of adoptive cell therapies [e.g., chimeric antigen receptor (CAR) T cells].

Master regulators of T cell exhaustion remain largely unknown. Although transcriptional profiling has implicated the differential expression of multiple TFs in Tex subsets (e.g., T-bet, EOMES, TCF1, IRF4, BATF, TOX, NR4A), some of these factors also are essential for differentiation of functional effector and memory $CD8^+$ cells (145, 164–173). Indeed, single-cell analysis revealed a striking degree of similarity in the transcriptional signatures of T cell exhaustion and activation (174). These paradoxical findings indicate that transcriptional analysis alone cannot fully dissect master regulators of lineage commitment versus exhaustion.

As one example, TCR-induced NFAT drives opposing transcriptional programs in T cells. In the absence of AP-1 cooperation, NFAT initiates regulatory programs leading to a hyporesponsive state (28, 156, 157). The activation of NFAT also drives expression of the high mobility group box (HMG-box) TF TOX, which primes chromatin opening as effector T cells transition to $TCF1^+$ progenitor Tex cells (175–177). However, once established, TOX expression and its downstream programs are maintained in an NFAT-independent manner (177). The sustained upregulation of TOX in antigen-specific $CD8^+$ T cells during chronic viral infections is likely enforced by a unique DNA demethylation landscape at the *Tox* locus (176). Importantly, recent studies showed that genetic deletion of *Tox* initially enhances effector cytokine production in chronically stimulated $CD8^+$ T cells but eventually induces a massive loss of Tex cells (175–177). Notably, in TOX-deficient T cells, chromatin accessibility is increased at a cohort of T effector genes, such as *Gzmb*, *Klrg1*, *Zeb2*, and *Nr4a1*, with reduced accessibility observed at genes associated with Tex progenitor programs, including *Tcf7*, *Bach2*, and *Ikzf2* (177). These data indicate that targeting TOX may not be an effective therapeutic approach, given its counteractive functions in exhaustion and survival.

In addition to its role in modulating TOX, NFAT induces expression of the NR4A nuclear receptor family of TFs in hyporesponsive T cells. Although the molecular mechanisms integrating these regulatory activities are still unfolding, published studies highlight the potential for altering TF expression to unleash a T cell response against cancer. Illustrating this point, deletion of NR4A in CAR T cell models limited tumor growth, presumably via its ability to enhance chromatin accessibility at T effector REs enriched for bZIP and Rel/NF-κB motifs (178, 179). On the flip side, during chronic LCMV infection, BACH2 epigenetically repressed regulatory programs for terminal exhaustion while promoting those for stem-like $CD8^+$ T cell differentiation (180).

Intriguingly, the concept of dysfunctionality or exhaustion is no longer confined to T cells, with similar states reported for NK cells and ILC2s, albeit the underlying molecular mechanisms remain obscure (181, 182). Chronic viral infection and tumor studies have highlighted that dysfunctional NK cells have limited cytotoxic activity, elevated expression of inhibitory molecules, lower levels of activating receptors, and impaired cytokine production (181, 183). Similar to T cell dysfunction, these effects rely upon epigenetic reprogramming. Genome-wide profiles of human NK cells isolated from CMV-infected individuals, which were chronically cultured ex vivo with NKG2C-specific antibodies, revealed dramatic changes in DNA methylation. Notably, the promoters of *LAG3*, *PDCD1*, and *TIGIT*, which encode inhibitory molecules and are associated with the PD-1 signaling pathway, and genes like *IKZF3*, *NFATC2*, *TOX*, and *ZBTB38*, encoding TFs and epigenetic regulators, were hypomethylated compared with control NK cells from CMV-negative sources (181). Similarly, Tex cells maintain a demethylated state at the locus, which encodes PD-1, during chronic viral infections, including LCMV and HIV infections (184). Even after diminishing viral load, *PDCD1* remains unmethylated, allowing Tex cells to rapidly reexpress high levels of PD-1 upon TCR restimulation. Given the growing importance of NK and $CD8^+$ T cells in cancer therapies, continued studies to identify key regulatory circuits controlling exhaustion-associated genes may provide new opportunities for improving their efficacies.

LOOKING TO THE FUTURE

The advent of single-cell expression and chromatin-based assays is revolutionizing how we understand immune cell identity and heterogeneity. These techniques were originally limited to transcriptome studies, but single-cell microfluidics have recently been commercialized as easy-to-use kits, which has democratized the utility of this approach. Processed data from single-cell approaches, be they transcriptomes or epigenomes, can computationally identify new cell populations, impute branch points in cell fates, and deconvolute heterogeneity as it arises during development or disease, in terms of both discrete cell types and distinct states of a given cell type (some refer to the latter as metacells) (124).

In this context, single-cell transcriptomics and accessibility landscapes have fundamentally challenged the notion of discrete functional lymphoid subsets. For example, while Th cell subsets generated ex vivo by strong polarizing cytokines resolve as distinct clusters, when Th cells are characterized in *Salmonella* (type 1 polarizing), *Nippostrongylus* (type 2 polarizing), or *Citrobacter* (type 3 polarizing) pathogen models by expression or motif imprinting of their TFs, single-cell RNA sequencing (scRNA-seq) and scATAC-seq reveal a continuum of helper cell populations along each polarization pathway (185). Within this functional spectrum exist cells that have regulatory landscapes and programs merging all three Th subsets. In addition to mouse infection models, experimental asthma produces a continuum of Th1 and Th2 programs within lungs, and analysis of inflamed human gut resolves ILC1s and ILC3s as a gradient of programs (108, 186). One caveat to acknowledge when considering the lack of clear functional subsets is the inherent biases of single-cell omics, stemming from low cell numbers in scarce states or incomplete representation of all genes in expression data. However, proteome-based analyses, like cytometry by time of flight (CyTOF), of T effectors generated in large numbers during in vivo infections again yield a continuum of cells (97). Thus, it appears that infection and inflammation can direct regulatory programs for cells within a given microenvironment to converge on a functional phenotype, presumably to either clear an infection or repair inflammation-induced damage.

In terms of deciphering gene regulatory circuits within cell states, the most exciting approaches to emerge are multi-omics, which combine expression and chromatin profiling at the single-cell level of resolution. For example, cellular indexing of transcriptomes and epitopes by

sequencing (CITE-seq) combines RNA and protein expression (via sequence-tagged antibodies), which is useful to categorize cell types based on signature protein markers whose mRNAs are expressed at minimal steady-state levels and often drop out at the lower depth of scRNA-seq (187). One of the most commonly used multi-omics platforms is now scRNA-ATAC, which will be extremely helpful for dissecting regulatory circuits in rare cell types or states, as well as identifying enhancers that are important for transitions between states (188, 189). As the transposase-based CUT-and-Tag (cleavage under targets and tagmentation) technique improves, it may one day be possible to examine chromatin binding profiles of nuclear factors at a single-cell level (190). In this context, a determination of the TF networks that cement cell states or facilitate transitions between them will benefit from data that emerge from perturb-seq approaches, which use CRISPR methodologies, coupled with either scRNA-seq or ATAC-seq (187, 191). Of course, the complexity of data sets from these techniques, and their analyses, is increasing dramatically. Continual progress in machine learning methods will go hand-in-hand with technical advances as we continue our journey to unravel the intricacies of circuits that drive expression programs in our immune systems, especially those that provide the requisite flexibility to face an ever-evolving universe of pathogens. Such insights will drive advances in the engineering of cellular therapeutics to activate or repress expression modules for more efficacious treatments of cancer, infectious diseases, and chronic inflammation.

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