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DNA methylation and the expanding epigenetics of T cell lineage commitment

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Abstract

During their development from progenitors, lymphocytes make a series of cell fate decisions. These decisions reflect and require changes in overall programs of gene expression. To maintain cellular identity, programs of gene expression must be iterated through mitosis in a heritable manner by epigenetic processes, which include DNA methylation, methyl-CpG- binding proteins, histone modifications, transcription factors and higher order chromatin structure. Current evidence is consistent with the notion that DNA methylation acts in concert with other epigenetic processes to limit the probability of aberrant gene expression and to stabilize, rather than to initiate, cell fate decisions. In particular, DNA methylation appears to be a non-redundant repressor of CD8 expression in TCR-γδ T cells and Th2 cytokine expression in Th1 and CD8 T cells, and is required to enforce clonally restricted Ly49 and KIR gene expression in NK cells. However, most of our knowledge is derived from in vitro studies, and the importance of DNA methylation in memory cell lineage fidelity in vivo remains to be shown convincingly.

Keywords: Epigenetic; DNA methylation; Histone; Chromatin; T cell

1. Introduction

During their development from bone marrow progenitors, B and T lymphocytes make a series of sequential, largely binary and irrevocable cell fate choices – T cell or B cell lineage, then in T cells between T cell receptor (TCR)γδ versus TCRαβ, CD4 versus CD8, and in CD4 T cells – Th1 versus Th2-lineages. With the exception of TCRγδ versus TCRαβ rearrangement, which reflects a change in the primary genetic information (i.e., the sequence of bases) within the cell, these cell fate choices are initiated and maintained without a change in DNA sequence-encoded information. Rather, each of the other lineage choices requires that cells transcribe (or enable the activation-induced transcription of) specific sets of genes, while repressing or silencing others. These changes in gene expression are encoded not by permanent changes in the primary genetic information of the cell but by changes in epigenetically encoded information that determines which genes are expressed.

Transcription is controlled in part by the assembly of relevant complexes of transcription factors on regulatory regions of genes. Thus, abundance, post-translational modifications, localization and higher order interactions with other transcription factors, co-activators or repressors are one set of mechanisms through which transcription is regulated. A second level of control is provided by epigenetic processes that affect the accessibility of transcription factors to the regulatory regions of their target genes within a highly ordered chromatin structure. Thus, by modulating the ease or probability with which transcription factors can engage the regulatory regions of genes and recruit RNA polymerase II containing complexes, epigenetic factors may silence, dampen or facilitate efficient transcription [1–8].

In principle, heritable programs of gene expression that are necessary to dictate the proper function of a specific cell type could be maintained solely by the continued expression of the same transcription factor(s) used to initially establish the specific program. Alternatively, heritability could be maintained if the initiating transcription factor(s) induced a stable

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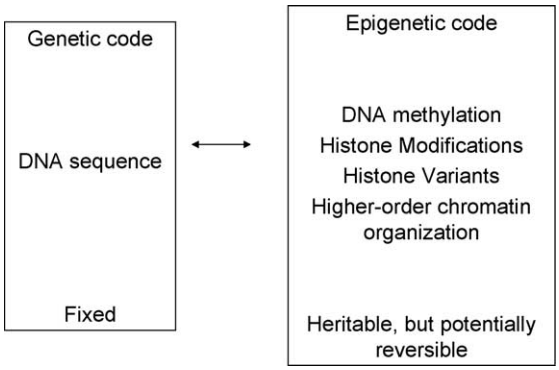

Fig. 1. Features of the genetic code compared to the epigenetic code.

epigenetic imprint (memory) of the gene expression program that could be transmitted through S phase from parental cells to their progeny in the absence of the factor(s) required to initiate these changes. In mammals, epigenetic information is encoded by variation in the methylation of DNA on cytosines, differences in the composition of histone proteins and post-translational modifications of histones associated with DNA in chromatin, and higher order chromatin structure.

Epigenetic regulation of gene expression is one of the most active areas in biology with >2000 publications in the past year. Moreover, the importance of epigenetic events in the development and proper function of the immune system is receiving increasing attention, and DNA methylation is the most well defined epigenetic mechanism for heritable propagation of epigenetic information. In this review, we will emphasize recent developments regarding the role of DNA methylation in the field of immunology. Important advances in the area of histone and chromatin modifications, how these modifications affect and are affected by differences in DNA methylation, and how these processes are integrated to achieve the proper balance between stability and plasticity of gene expression needed to maintain fidelity of T cell fate and function, while responding to environmental cues, will be a second focus (Fig. 1). We will discuss epigenetic regulation in the context of key differentiation processes in immunology, including T lymphocyte lineage development in the thymus and T cell function polarization in the periphery. Other reviews in this issue will address important epigenetic influences on B cell development and antibody responses. We apologize in advance to any authors of primary research papers who, because of space limitations, are not cited directly.

2. Epigenetic mechanisms of gene silencing

2.1. DNA methylation

In mammals, methylation of DNA on cytosine residues occurs almost solely in the context of symmetrical CpG dinucleotides. The maintenance methyltransferase, DNA methyltransferase 1 (Dnmt1) is upregulated during S phase, recruited to DNA replication forks by proliferating cell nuclear antigen (PCNA), and preferentially active on hemimethylated CpG substrates, such as those generated during replication of symmetrically methylated CpG sites [9]. By methylating CpG sites on new daughter strands, Dnmt1 maintains methylation patterns that are complementary to those of the parental strand (Fig. 2). In doing so, Dnmt1

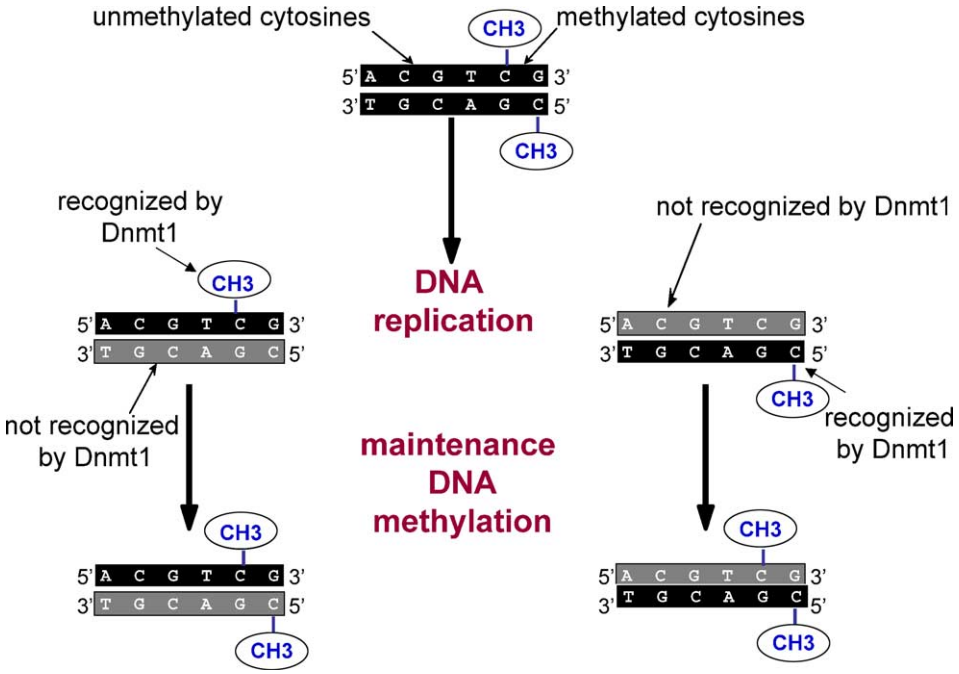

Fig. 2. DNA methyltransferase 1 (Dnmt1) maintains DNA methylation patterns through S phase by copying the pattern of CpG methylation from the parental strand to the progeny strand.

copies, with high but imperfect fidelity, the pattern of cytosine methylation in the DNA of parental cells to the DNA of their progeny in a manner conducive to the heritable propagation of information needed to assure consistency of cell fate and function. There are two other enzymatically active DNA methyltransferases, Dnmt3a and Dnmt3b, which do not prefer hemi-methylated CpG substrates and can catalyze de novo methylation of unmodified CpG sites [10]. Some of the pathways influencing Dnmt1 may also affect Dnmt3a and Dnmt3b since the distinction between maintenance and de novo methylation is not absolute. There is evidence for a role for Dnmt3a and/or Dnmt3b in the maintenance methylation of high density CpG sequences and, conversely, Dnmt1 can cooperate with Dnmt3a and/or Dnmt3b in de novo methylation in some settings. For example, there is cooperation between maintenance and de novo methylation during methylation spreading where a focus of methylated CpG sites is extended to yield longer sequences of contiguously methylated DNA [11]. Each of these Dnmts undergoes alternative splicing to create distinct isoforms that appear to vary in function and patterns of tissue expression, adding additional complexity to the control of DNA methylation [12].

The counterpoint to maintenance methylation, de novo methylation and methylation spreading is demethylation [13]. Demethylation of methylated CpG sites can occur passively during DNA replication if Dnmt1 levels are insufficient, if Dnmt1 loses fidelity, or if Dnmt1 is excluded from replication forks. Inaccurately copied parental DNA methylation sites generate hemimethylated CpG sites in progeny DNA strands that can become fully demethylated after additional DNA replication. Passive demethylation via protection from, or displacement of, the maintenance and de novo methyltransferase machinery has been described for CpG-rich islands. For example, the Sp1 transcription factor can bind to its consensus site even if methylated, to sterically exclude methyltransferases and induce constitutive transcription [14]. There are numerous reports of active demethylation, sometimes within hours of stimulation or without cell division, and this has been an area of intense investigation for >20 years [15]. Examples include demethylation of DNA in the male pronucleus of the fertilized egg [5], of the brain derived neurotrophic factor (BDNF) promoter in primary neuronal cells in response to depolarization in vitro [16,17], and of the IL-2 promoter in activated primary T cells [18]. However, there is as yet no clearly defined molecular entity responsible for enzymatic demethylation of symmetrically methylated CpG sites in non-dividing mammalian cells.

DNA methylation can repress gene expression through direct mechanisms, such as blocking the binding of some transcription factors that have CpG sites in their recognition motifs [19]. Indirect mechanisms have more broad regulatory potential and include interacting with methyl-CpG-binding proteins, recruiting histone deacetylases and histone methyltransferases, and nucleating other processes involved in the formation of compact chromatin. All of these can render a locus less accessible or stably inaccessible to the transcription apparatus (see below).

2.2. Methyl-CpG-binding proteins

Multiple proteins with affinity for sequences containing methylated cytosines have been described, of which the most important for regulation of mammalian genes appear to be MeCP2, MBD1 and MBD2 [20]. MeCP2 can bind to a single symmetrically methylated CpG site in vitro. MBD1 and MBD2 possess homologous methyl-CpG-binding domains and exhibit similar binding properties, although MBD1 can also associate with non-methylated DNA [21], while MBD2 may require multiple methylated CpG sites to bind effectively [22]. The interaction partners and functions of these proteins are manifold, incompletely understood, and beyond the scope of this review. However, the following working model is sufficient for the topics to be covered below. A methyl-CpG-binding protein can bind methylated DNA, directly compete with or displace other DNA-binding proteins, and repress transcription from methylated promoters. Repression can also occur indirectly through recruitment of other protein complexes to methylated DNA. MeCP2, MBD1 and MBD2 can recruit histone deacetylase and histone methyltransferase complexes, and thereby, localize modifications in chromatin structure. Further associations between MeCP2/MBD2 and Dnmt1, and between DNA methyltransferases and histone modifying complexes, reinforce this recruitment and localization. Methyl-CpG-binding proteins may, thus, triangulate with two key sets of epigenetic machinery: DNA methylation and histone modification. By contrast to the relatively promiscuous binding of these proteins to methylated CpG in naked DNA, they are bound to only a fraction of methylated CpG in situ. While this may relate to limitations in the abundance of methyl-CpG-binding proteins relative to methylated CpG, their distribution appears not to be random, but how methyl-CpG-binding proteins are targeted to or away from specific gene sequences is poorly understood. Mechanisms may include interaction of methyl-CpG-binding proteins with partners that convey sequence-specificity [23] or post-translational modifications. Notably, depolarization of neurons not only can cause a rapid reduction in DNA methylation in the BDNF promoter as noted above, but calcium-dependent phosphorylation of MeCP2, dissociation of MeCP2 and its associated repressor complex proteins from promoter sequences, and transcription of the BDNF gene [16,17]; whether phosphorylation and disassociation of MeCP2 is causally linked to DNA demethylation (or vice versa) is at present unclear. Nonetheless, these findings, and recent findings in the field of cytokine gene regulation (discussed below), indicate that classical signaling pathways can lead to rapid epigenetic modifications in non-dividing cells. Evidence for the relevance of this to other cell types, signaling pathways, and/or genes, is eagerly awaited.

2.3. Post-translational histone modifications and histone variants

Nucleosomes are comprised of ~160 bp of DNA wrapped around a core of eight histones, consisting of two copies each of H2A, H2B, H3 and H4 (for an animated view see http://www-u.life.uiuc.edu/%7Ej-roland/artwork.html). Neighboring nucleosomes are connected by short segments (<80 bp) of DNA and compacted by the linker histone H1 [24]. Tails of histone proteins protrude from the nucleosome and are sites of post-translational modifications [25]. With multiple modifications possible for each histone molecule and multiple histones in each stretch of nucleosomal DNA, the combinatorial potential for epigenetic modification is high and has been termed the "histone code" [26]. The many contexts and interpretations of this code are yet to be deciphered, however, the following are some features that appear to be consistent across several genes and mammalian cell types. Acetylation of histone H3 on lysine residues 9 (H3K9-Ac), 14 (H3K14-Ac) or 18 (H3K18-Ac), and/or of histone H4 on lysine residues 5 (H4K5-Ac) or 8 (H4K8-Ac), is associated with "open" accessible chromatin and active gene expression. Lysine residues can also be modified by the addition of one, two or three methyl groups. Methylation of histone H3 on lysine residue 4 (H3K4-Me) is also associated with active expression, but unlike H3 acetylation, may persist longer after transcription ceases providing a mark of transcriptional competence [27]. Conversely, methylation of histone H3 on lysine residues 9 (H3K9-Me) or 27 (H3K27-Me) is associated with "closed" condensed chromatin and repressed or silenced gene expression, and stable repression of genes found in heterochromatin is associated with genes that are tri-methylated on lysine 9 [3,28]. Histone imprints interact with each other. For example, methylation of H3K9 precludes acetylation at this residue, and phosphorylation of H3S10 appears to facilitate other transcriptionally favorable H3 modifications—acetylation of H3K9 and methylation of H3K4. In addition, there are many other histone modifications, including acetylation or methylation or phosphorylation at other sites, ubiquitination and ADP-ribosylation, whose functions are less well characterized or generalized.

Histone acetylation is a dynamic process, since histone acetyl transferases (HATs) and deacetylases (HDACs) can rapidly add or remove acetyl groups, respectively. By contrast, histone methylation appears to be a more stable modification, which is consistent with the role of H3K9 and K3K27 methylation in gene silencing and the absence, to date, of demethylases that can catalyze the removal of methyl groups from lysine residues. However, recent evidence that methylated arginine residues of H3 and H4 can be removed by peptidyl arginine deiminase 4 [29,30] indicates that at least some forms of histone methylation are reversible. Moreover, while histone methylation on H3K9 is commonly associated with stable repression of genes in heterochromatin, H3K9-Me is also found in euchromatin where it may play a more dynamic role in gene regulation. For example, the rapid induction of IFN-β in response to virus infection is associated with acetylation of H3K9, but transcription is terminated within 12 h. Termination of IFN-β transcription is due in part to sequential HDAC-mediated deacetylation followed by methylation of H3K9 by the histone methyltransferase G9a [31].

In addition to post-translational histone modifications, a number of variant histones may be used in the place of the canonical histones noted above and in turn affect transcriptional competence and chromatin structure. For example, one variant of H2A, H2AX, is incorporated in nucleosomes at sites of double-stranded DNA breaks and plays a critical role in immunoglobulin class switch recombination [32]. H3.3 is a variant of H3 [33]. While H3 is incorporated into nucleosomes during S phase in a replication-dependent manner, H3.3 may either be incorporated during S phase or exchanged for H3 in a replication-independent manner [34,35]. Moreover, compared to H3, H3.3 is enriched for transcriptionally favorable histone modifications such as H3-K9Ac, at least in flies [36]. Thus, there are two mechanisms by which nucleosomes containing H3 with repressive modifications like H3-K9Me can be replaced by ones with favorable modifications: during S phase when DNA is replicated and by replication-independent exchange of H3.3 for H3 at other times. The latter mechanism was recently shown to participate in Pax5-dependent replacement of H3 methylated on lysine 9 with H3.3 lacking this modification at sites in the immunoglobulin heavy chain locus undergoing V(D)J recombination [37].

2.4. Nucleosome assembly, higher-order chromatin structure and chromatin remodeling

As noted above, histone octamers must be assembled on the parental and daughter strands of DNA during S phase. Whether this is accomplished by assembly of nucleosomes containing exclusively the original histone octamer on the parental strand and newly incorporated histones on the daughter strand or by semiconservative assembly with half new and half original histones on each strand is unclear [35]. Once assembled on DNA, nucleosomes provide a barrier to the transcriptional machinery, so the association of DNA with histone octamers must be altered to facilitate the binding of transcription factors and transit of the RNA polymerase complex. As a consequence, specific sites within genes that are actively transcribed or poised for transcription in response to environmental signals (e.g., through the TCR) can be identified by their accessibility to endonuclease cleavage by DNase I, so-called DNase I hypersensitivity sites, or by restriction enzymes. Often, but not always, these sites correlate with regulatory sequences involved in activating gene expression, including promoters, enhancers and locus control regions. Genome-wide mapping of such sites has been recently reported for the K562 cell line [38], however, as for all epigenetic modifications, there is likely to be a high level of cell- and stimulus-specificity that will necessitate more detailed mapping in purified primary cells at specific stages of differentiation (see below).

Alteration of nucleosome–DNA interactions is mediated by ATP-dependent, macromolecular chromatin remodeling complexes, which may slide, roll, reposition, and in some cases, remove and reassemble the histone octamer [39]. There appears to be substantial diversity in the composition and specific functions of octamers between cell types, including T and B lymphocytes (reviewed in [40]). Interestingly, chromatin remodeling complexes containing SNF2-family ATPases are required for proper regulation of DNA methylation and H3K4 methylation as well. For example, cells from mice lacking the lymphocyte-specific helicase (Lsh) show global DNA demethylation and selective enrichment of trimethylated H3K4 'without a change in methylated H3K9' at pericentromeric heterochromatin [41,42].

DNA wrapped around nucleosomes can be compacted into a beads-on-a-string conformation approximately 10 nM in size, and thence into a zigzag array of nucleosomes to form a structure known as the 30 nM chromatin fiber [43]. Chromatin fibers, in turn, may be compacted further into higher-order structures and then positioned within the nucleus into either "open" areas of euchromatin or "closed" areas of condensed heterochromatin. Much is being learned about these structures courtesy of advanced molecular, biophysical and imaging techniques. For the purposes of this review, it is worth highlighting three aspects: (a) nucleosome condensation into nM-sized structures is highly dependent on the linker histone H1 and this histone has an affinity for methylated CpG sites [44], (b) heterochromatin formation is dependent on heterochromatin protein 1 (HP1α and β), that can bind to themselves, Dnmt1, Dnmt3a, histone methyltransferases, and the H3K9-Me product of the latter enzymes [45–47], and (c) the intranuclear localization of genes to heterochromatin is associated with repression or silenced expression while activation of gene expression is associated with relocalization to euchromatin [3].

2.5. Interaction, integration and targeting of DNA methylation and other epigenetic modifications to specific regions and genes

For most genes and cell types, DNA methylation, histone modifications and higher order chromatin structure are unlikely to operate independently. During short-term induction or repression of gene expression, changes in histone modifications are more likely to provide one level of control that may be subservient to, or dominant over, other changes in DNA methylation, methyl-CpG-binding protein localization and other epigenetic elements (see below). In some cases this short-term subservience or dominance may be real, in other cases it may reflect shortcomings in the snapshot and/or population-based analytical methods used. During long-term regulation of gene expression, it is more clear that modified histones usually act in concert with changes in DNA methylation and other chromatin-associated factors to yield epigenetically stable programs of gene expression, but how these processes are linked and how and to what extent faithful inheritance of specific patterns of modified histones are assured are ill-defined [48].

If the assembly of histone octamers during S phase occurs through a semiconservative mechanism in which histones from the parental strand are divided and shared by the parental and daughter DNA strands, this could provide a platform for the heritable propagation of histone composition and modifications through S phase, if a mechanism for reading and copying of patterns existed analogous to the mechanism by which Dnmt1 copies the pattern of CpG methylation [39]. Whether histone assembly occurs via this semiconservative mechanism is unclear, but recently a mechanism for copying has been suggested in which DNA methylation and the methyl-CpG-binding protein MBD1 directs H3K9 methylation in human cells. MBD1 (and MeCP2 but not MBD2) were shown to associate with the H3K9 methyltransferase SETB1, and this complex was recruited to the replication fork by PCNA, as is Dnmt1. This resulted in the linked copying of the methylated CpG pattern, binding of MBD1 to methylated CpG and methylation of H3K9 at the p53 binding protein 2 gene [49]. The extent to which this finding can be generalized is as yet uncertain, and as alluded to above, what determines which, if any, methyl-CpG-binding protein associate with methyl CpG at specific sites is an area of active interest. Conversely, while MBD2 does not associate with SETB1, it does associate with MBD3 and Dnmt1 at the replication fork in late S phase [50]. Since MBD2 does associate with HDACs, this may provide a mechanism by which some regions of the genome are targeted for DNA methylation and histone deacetylation but not H3K9 methylation, as is the case at the IL-4/IL-13 locus in naïve T cells [51].

Another major unanswered question in the field is how de novo DNA methylation and repressive histone modifications are targeted to specific sites to induce transcriptional silencing. One report using promyelocytic leukemia cells described a retinoic acid receptor/PML fusion protein that associates with Dnmt1 and Dnmt3a and targets DNA hypermethylation and aberrant silencing to retinoic acid response genes [52]. But the most exciting recent information is related to a series of publications indicating a role for non-coding RNAs in silencing. An antisense RNA transcript may be involved in targeted DNA methylation and inherited silencing of a human alpha-globin gene, although Dnmt recruitment was not examined in this case [53]. Also, it was recently shown that Dicer, which generates small interfering RNAs (siRNAs), is required for the formation of stable heterochromatin in chicken-mouse cell hybrids; while this suggests that siRNAs are required to direct DNA and histone methylation to regions destined to become constitutive heterochromatin, this was not evaluated in this study [54]. Two recent reports show that artificial siRNAs targeted to CpG containing promoters in human cells can induce the recruitment of Dnmt1 and Dnmt3b, de novo DNA methylation, H3K9 methylation and transcriptional silencing [55,56]. What remains to be determined is whether endogenous microRNAs or siRNAs are involved in the targeted silencing of specific genes in a physi-

ological manner. Similarly, how DNA demethylation, passive or active, is directed to specific regions is as yet unknown, although tantalizing data regarding demethylation in T cells is discussed below.

3. Probability, heritability, and plasticity

The above description of the machinery of epigenetic regulation is potentially misinterpretable as a linear series of on–off switches, or a static set of positive and negative building blocks that are arithmetically summed, or a kinetic progression of qualitative influences on all-or-none decisions. The complex reality is that epigenetic mechanisms appear to act more as tunable thresholds or rheostats that facilitate or antagonize the actions of transcriptional activators and repressors (Fig. 3). There is a probability associated with each DNA methylation, histone modification and chromatin remodeling reaction and this may vary between alleles of the same gene, between different genes, within an individual cell according to the phase of the cell cycle, between individual cells depending on their current stimuli or previous experiences, and between different cell types. This makes the interpretation of cell population data challenging. Nevertheless, some quantitative features of epigenetic regulation are emerging and worth monitoring for future developments.

Estimates of the fidelity of Dnmt1 maintenance methylation have been made using new technology, with good agreement between predicted and observed data [57]. For most CpG sites this fidelity appears to be an extraordinarily high ≥99%, especially given that there is no known proof-reading machinery for DNA methylation. If methylation-based epigenetic fidelity is dependent on patterns rather than individual CpG sites, these data are probably sufficient to explain faithful inheritance through hundreds of cell divisions, and thus, the lifespan of most cells in the body. However, such estimates have not yet been made under conditions of rapid cell division where Dnmt1 may be limiting, such as in T and B cells responding to antigen in vivo. We also lack estimates for Dnmt3a and Dnmt3b processivity under steady-state or limiting conditions for de novo methylation. Consequently, we do not yet know whether the methylation underlying a repressed state is inherited with more fidelity than the demethylation underlying a primed state, or vice versa.

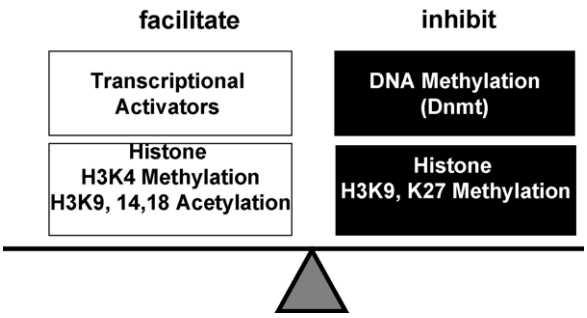

Fig. 3. Transcription is controlled both by the abundance of transcription factors and by epigenetic factors that affect the ability of transcriptional factors to bind to their target sites in chromatin (i.e., accessibility). For purposes of simplicity, transcription factors that activate transcription are shown but transcriptional repressors are not, and of the many different histones and histone modifications that encode information affecting transcription, only H3 and a subset of its modifications are shown.

The thresholds for methylation-mediated effects are low: the average density of methylated CpG sites in vertebrate genomes is ∼1 mCpG per 100 bp and this is sufficient to repress weak endogenous promoters [58]. Experimentally, methylation levels as low as ∼1 mCpG per 300 bp can significantly but incompletely repress episomal reporter genes in cell lines [59]. The numbers of MeCP2 molecules per cell suggest that this key factor may be limiting. At ∼4,000,000 molecules per nucleus, calculated from mouse brain and kidney tissue, DNA coverage has been estimated to be ∼1 MeCP2 molecule per 5–20 nucleosomes [60], and the abundance of other methyl-CpG-binding proteins may also be limiting [61]. In contrast, histone H1 is more abundant and probably sufficient to cover any exposed internucleosomal methylated CpG sites. There are currently no quantitative estimates of the levels or half-lives of specifically modified histones, even for acetylated histones where several histone acetylase and deacetylase enzymes have been well characterized. Attempts are being made at quantifying the levels of HP1 and the rates of gene heterochromatinization. Recent studies suggest that there may be unexpected dynamism and plasticity at this level with much HP1 exchange and traffic in the nuclei of living cells, including lymphocytes [62,63].

These estimates cumulatively suggest that, at equilibrium, the multi-layered epigenetic machinery of a cell is probably sufficient to maintain repressed expression across almost the entire genome, with inheritance and fidelity potentials capable of covering the division lifespan of the average cell. Perhaps accordingly, transcriptional noise or “random” gene expression has been estimated at <1 aberrant transcript per 10,000 cells for test genes such as beta-globin in non-erythroid cell lines (by extrapolation, <10 aberrant transcripts per mammalian genome at any one time) [58]. When epigenetic repression is completely removed, the overall dynamic range for an average inducible gene has been estimated at ∼25,000 fold [61], which appears to match recent observations of endogenous genes in lymphocytes during their differentiation and function (see below).

4. Specific examples in the immune system

4.1. T cell development in the thymus

Dnmt1 is required for proper maturation of thymic progenitors as shown from studies in T cell-specific conditional Dnmt1 knockout mice [64]. Conditional deletion of Dnmt1 in early double-negative (DN) thymocytes is followed by cellular and DNA replication and DNA demethylation in response to β-selection at the DN3 stage, resulting in marked DNA

demethylation at the double-positive (DP) and later stages. This is associated with a profound reduction in cells of the TCRαβ-lineage at the DP, single-positive (SP) and mature T cell stages. This results from apoptosis of cells as levels of DNA methylation fall below a critical threshold, which occurs in all mammalian cell lineages studied to date (with the exception of undifferentiated ES cells and rare tumor cell lines) [64–66]. In addition to the attrition of TCRαβ-lineage cells, the numbers of TCRγδ cells is increased in these mice, which appears to reflect in part their more limited intrathymic replication and, as a consequence, less profound DNA demethylation. A similar, though less severe, attrition of TCRαβ lineage cells is seen in thymocytes lacking the lymphocyte-specific helicase (Lsh), in association with markedly reduced and aberrant DNA methylation [41,67,68].

4.1.1. *TCRαβ versus TCRγδ*

The DNA of TCR loci is methylated in double-negative thymocytes. Activation of the TCRβ gene proceeds through a pathway involving IL-7Rα signaling, emergence of DNaseI hypersensitivity sites, and demethylation of CpG sites [69–72]. These events open the locus to the V(D)J recombination machinery, and allow expression of the TCRβ chain as part of a pre-TCR complex [73]. Pre-TCR signaling triggers ATP-dependent BAF chromatin remodeling complexes that affect expression of multiple survival and differentiation genes, as well as excluding expression of the other TCRβ allele [74]. Excluded alleles are characterized by DNA hypermethylation and compact chromatin but the repressed intermediates preceding these silenced states have not yet been dissected. It is noteworthy that targeting of a histone H3K9 methyltransferase to an artificial TCRβ minilocus can induce such features, and render TCR genes inaccessible, although this plausible mechanism has not yet been examined in the context of the endogenous TCRβ locus or shown to affect allelic exclusion [75]. Similar processes may occur during recombination at the less well studied TCRγ locus [76]. In comparison, allelic exclusion of TCRαδ loci is weaker, perhaps because of potent enhancers that regulate locus-specific histone modification and demethylation [73,77].

Failure of TCRγ rearrangement in IL-7Rα knockout mice is associated with persistent DNA methylation at the TCRγ enhancer, suggesting that DNA demethylation is either necessary for rearrangement or occurs as a consequence of other processes that are essential [70]. However, Dnmt1-deficient thymocytes exhibit normal V(D)J recombination and allelic exclusion [8,64]. This does not exclude the possibility that demethylation is necessary for recombination to occur, but does suggest that DNA demethylation is not a limiting factor in V(D)J recombination nor are proper levels of DNA methylation required to enforce allelic exclusion. V(D)J recombination at the TCRα, β and γ and immunoglobulin heavy chain loci is also not impaired in the absence of Lsh [67].

The irreversible silencing of terminal transferase expression in mature thymocytes is also relevant to T cell development. As described in greater detail in the accompanying paper by Smale and co-workers, their elegant time-course study has recently shown that silencing can occur within 8 h of removal of the maturation stimulus, and is associated with nucleosome remodeling, loss of H3K9-Ac and gain of H3K9-Me at the terminal transferase gene promoter [78]. Histone methylation in this setting initiates at the promoter then spreads across the gene at the remarkable rate of ∼2 kb/h (i.e., ∼1 nucleosome per 5 min) in normal thymocytes. Importantly, this rapid spreading did not occur in an abnormal cell line where the terminal transferase gene was only reversibly repressed but not irreversibly silenced. In the normal silencing scenario, heterochromatin relocalization of the terminal transferase gene was a relatively early event in thymocytes, while CpG DNA methylation was a relatively late event that continued to increase in mature T cells after emigration from the thymus to the spleen [78].

4.1.2. *CD4 versus CD8*

The CD4 gene is a target of reversible repression in double-negative thymocytes and then permanent silencing as double-positive cells differentiate towards the single-positive $CD8^+$ state. This silencing is an epigenetically regulated event but DNA methylation does not appear to be necessary. Instead, there are specific repressive pathways in $CD8^+$ T cells mediated by Runx3 interacting in *cis* with a defined CD4 silencer sequence [79]. The silencing machinery triggered by Runx3 may involve other silencer-binding factors [80], histone deacetylation [81], BAF chromatin remodeling complexes [82], and/or heterochromatin HP1 [83]. Significantly, the inheritance of these epigenetic modifications has sufficient fidelity to be maintained even after removal of the instigating silencer [84]. This process remains the prototypic permanent silencing event for an endogenous gene during normal cell differentiation [85,86]. An equivalent silencing process for shutting down CD8 gene expression in $CD4^+$ T cells has not been described.

Demethylation of the CD8α and CD8β genes occurs during thymocyte maturation from the double-negative to the $CD4^+CD8^+$ stage. The retention of demethylated CD8 genes in single-positive $CD4^+$ cells provided early evidence for the notion that these cells are derived from previously $CD8^+$ precursors [87,88], which was later proved by lineage tracing studies. A role for DNA methylation in regulation of CD8 expression was also seen in conditional $Dnmt1^{-/-}$ mice [64], in which aberrant CD8α and CD8β expression occurred in a large fraction of TCRγδ lineage cells. De novo remethylation of the CD8α gene has been associated with the death of misselected thymic emigrants [89]. However, other mechanisms must be able to silence the CD8 gene after commitment to the CD4 lineage, since double-positive $CD4^+CD8^+$ T cells are not found in the periphery of conditional $Dnmt1^{-/-}$ mice [64]. Repression by the transcription factor Ikaros was a candidate mechanism, but other studies suggest a role for Ikaros and its relative Aiolos in CD8α gene activation rather than silencing [90]. An alternative candidate is GATA3 since overexpression of this transcription fac-

tor can inhibit $CD8^+$ single-positive thymocyte development while expression of the repressor of GATA3 (ROG) increases the levels of $CD8^+CD4^{neg}$ thymocytes [91]. While a direct mechanistic link between GATA3 and epigenetic regulation of CD8 expression has not yet been defined, the interaction between GATA3 and a methyl-CpG-binding protein can play an important role in controlling IL-4 expression in T cells (discussed in Section 4.2.3 below).

4.2. T cell function

4.2.1. IL-2

A role for DNA methylation in the regulation of T cell function was suggested by early studies in which the expression of IL-2 was shown to be enhanced by agents that inhibit DNA methylation [92]. Subsequent studies have shown that this gene is quiescent, the IL-2 promoter DNA is methylated, and the locus is contained in inactive chromatin in resting naïve T cells [18,93]. Early after TCR-dependent activation, one or both alleles become activated, depending on the strength of stimulus [94,95]. Although IL-2 expression occurs in the absence of cell division, cell division increases the probability and frequency of these events [96–98]. In naïve CD4 and CD8 T cells from conditional $Dnmt1^{-/-}$ mice, IL-2 expression is increased ∼five-fold following activation in vitro for 24–72 h [99]. This relatively modest increase may reflect the fact that the IL-2 gene normally undergoes rapid DNA demethylation in wild-type cells in response to activation. Remarkably, demethylation in the IL-2 promoter appears to commence within hours after T cell stimulation in vitro, within 20 min of T cell stimulation in vivo, and well before entry of cells into S phase. This suggests the occurrence of active, catalytic DNA demethylation targeted to a specific subset of CpG within ∼800 bp of the IL-2 transcription initiation site [18]. These studies also showed that methylation of these cytosines repressed IL-2 transcription, supporting the functional importance of demethylation for expression of IL-2.

Further support for the notion that active DNA demethylation may be targeted to specific regions in response to T cell activation, comes from recent studies of the Th2 cytokine locus, which are described below. If the findings of active demethylation in the IL-2 and IL-4 genes in response to T cell activation can be reproduced and the mechanism elucidated, it would represent a major advance since there are as yet no known enzymes that can catalyze DNA demethylation.

4.2.2. IFN-γ

IFN-γ was one of the first cytokines shown to be regulated epigenetically. A role for DNA methylation in the regulation of IFN-γ in T cells is supported by several lines of evidence: (a) augmented expression after treatment with DNA methylation inhibitors [100,101], (b) levels of DNA methylation in the promoter correlate inversely with differences in expression of IFN-γ between neonatal and adult or "naïve" and "memory" T cells [102–104] and between Th1 and Th2 cells [101,105], (c) heritability of a demethylated state in $CD8^+$ T cell clones correlates directly with expression [106], and (d) de novo methylation in T cell populations correlates with reduced expression [107]. The first of these features is faithfully reproduced in $Dnmt1^{-/-}$ T cells, in which expression of IFN-γ is increased ∼5–10-fold following activation in vitro for 24–72 h [99]. Increased IFN-γ expression appears to be primarily if not solely due to DNA demethylation in the IFN-γ gene and not due to increased expression of transcription factors known to facilitate IFN-γ expression (e.g., T-bet or eomesodermin) or to indirect *trans*-mediated effects on the IFN-γ promoter [51,64]. Consistent with findings in $Dnmt1^{-/-}$ T cells, naïve T cells lacking the methyl-CpG binding protein MBD2 produce increased amounts of IFN-γ in response to activation and before entering the cell cycle in the absence of cell division [108]; while likely to be a result of MBD2 binding to methylated CpG in regulatory regions of the IFN-γ gene and recruitment of repressive chromatin modifying complexes, this was not evaluated in this report.

While DNA demethylation in the IFN-γ promoter correlates with expression and constructs and transgenes containing the IFN-γ promoter can drive significant expression [109–111], intronic and distal elements accessible to DNaseI are required for full expression [112,113]. Agarwal and Rao defined DNase HS sites in the introns of the mouse IFN-γ gene that exhibited increased accessibility during Th1 differentiation [114], and the general regulatory role of accessibility was reinforced by in situ studies of the repositioning of the IFN-γ gene from heterochromatin to euchromatin in the nuclei of polarized Th1 or Th2 cells [115].

Recent studies have augmented our understanding in this area by characterizing epigenetic modifications in conserved nucleotide sequence elements around the IFN-γ gene. These modifications include DNA methylation [116], histone H3 and H4 acetylation [117–122] and histone H3K4-Me methylation [116,121]. The sequence elements involved extend in conservation across mouse, human, rat and dog genomes, and in distance >50 kb upstream and downstream of the IFN-γ exons. One significant conserved nucleotide sequence is an enhancer-like element, designated 5′CNS, −3.6IFN-G or IFNgCNS1, ∼5 kb upstream from the ATG start codon [116,118,121]. This element exhibits DNaseI hypersensitivity and DNA demethylation in Th1 cell lines, association with modified histones H3-Ac, H4-Ac and H3K4-Me, and is a target for the key transcription factors AP-1, NFAT1, Stat5, and T-bet. When conjugated to IFN-γ reporter constructs, IFNgCNS1 can also significantly enhance IFN-γ expression. Shnyreva et al. have recently defined another conserved nucleotide sequence, designated IFNgCNS2, ∼18 kb downstream of the IFN-γ ATG start codon [121]. This element exhibits enhancer-like activity when placed downstream of IFN-γ reporter constructs, DNase hypersensitivity in primary mouse Th1 and $CD8^+$ T cells, and association with modified histones H3-Ac, H4-Ac and H3K4-Me. Consistent with these

signs of modified chromatin, low level extragenic transcription was detected in this region, specifically in Th1 cells. Interestingly, in T-bet knockout $CD8^+$ T cells, significant histone modifications at IFNgCNS2 are still found, suggesting the potential for T-bet-independent chromatin remodeling at this site. It is not yet known whether this reflects the activity of another T-box family member such as eomesodermin [123] and/or a distinct pathway operative in $CD8^+$ but not $CD4^+$ T cells [124].

4.2.3. The Th2 cytokines—IL-4, IL-13 and IL-5

The regulation of IL-4 is perhaps better understood than that of IFN-γ. A cell-specific enhancer associated with a DNase hypersensitivity site in the second intron was defined soon after the discovery of this important interleukin [125]. Five DNase hypersensitivity sites across the mouse IL-4 gene were subsequently mapped and characterized for chromatin remodeling behavior during Th1/Th2 differentiation [126]. Around the same time, evidence for epigenetic regulation emerged from the demonstration of monoallelic expression of IL-4 in naïve $CD4^+$ T cells [127,128]. These findings increased in significance when this expression characteristic was found to extend in a coordinated manner across the IL-4, IL-13, IL-5 genes, and when this cluster of Th2 cytokines was shown to be coordinately regulated by nucleotide sequence elements conserved across several species [129–131]. CNS-1 (hereafter referred to as IL4CNS1) is ∼10 kb upstream of the IL-4 ATG start codon, between the IL-4 and IL-13 genes, and corresponds to the HSS2 and HSS3 DNase hypersensitivity sites identified by Flavell and co-workers [130,132]. CNS-2 (hereafter IL4CNS2) is ∼15 kb downstream of the IL-4 ATG start codon, between the IL-4 and KIF3A genes and corresponds to the DNase hypersensitivity site V defined by Rao and co-workers [114,130]. Deletion of IL4CNS1 in engineered mice has significant impact on IL-4 expression in T cells while deletion of IL4CNS2 affects IL-4 expression in both T cells and mast cells [131,133]. Recent studies from Flavell and colleagues indicate that regulatory regions extend further, including additional DNase hypersensitive sites within a putative locus control region located downstream of the IL-13 gene and within the 3′ portion of the adjacent and ubiquitously expressed RAD50 gene [132]. Moreover, they have shown that chromatin loops are formed in this region, such that the promoters of the IL-4, IL-13 and IL-5 genes are approximated to create a core configuration in all cell types, which is modified by the inclusion of the locus control region in T and NK cells but not other cell types [134]. However, in terms of epigenetic characterization, IL4CNS1, IL4CNS2 and proximal elements within the IL-4 gene have received the most study.

There are multiple components of epigenetic machinery that target these regulatory sequence elements in the IL-4/IL-13 locus. DNA methylation was identified as an important level of control of IL-4 gene expression, as for IFN-γ, through the mapping of CpG site methylation changes across the gene during T cell differentiation and the analysis of the effects of inhibiting Dnmt1 [64,96,135–137]. Makar et al. [51] using bisulfite sequencing showed that ∼70% of CpGs in the promoter and ∼90% of CpGs in IL4CNS1 and the IL-4 intronic enhancer are methylated in naïve $CD4^+$ and $CD8^+$ T cells, and that this degree of methylation is maintained by active recruitment of Dnmt1 to these regions when naïve T cells are activated in non-polarizing and Th1 conditions. Yet, CpGs in these regions are not fully methylated, there are no CpGs and so no methyl-CpGs within the proximal 250 bp of the IL-4 promoter, and the locus remains in poised euchromatin lacking the histone H3K9-Me methylation mark associated with stable gene silencing [51,115]. These features, and the residual expression of small amounts of GATA-3, may be important in allowing naïve $CD4^+$ T cells to express low levels of IL-4 shortly after activation.

During early Th2 differentiation, mapping studies suggest that DNA demethylation initiates in the IL-4 promoter, then occurs at IL4CNS1 and IL4CNS2, and then extends from the promoter downstream through the exons of the IL4 gene during later Th2 polarization [136]. However, DNA demethylation in these regions is not observed prior to cell replication and declines slowly over weeks, in association with the exclusion of Dnmt1 from these regions and the failure to copy the pattern of DNA methylation from parental to daughter DNA strands during cellular replication, i.e., passive demethylation [51,136]. Substantial demethylation lags behind the increase in IL-4 and IL-13 expression and the increase in histone acetylation in the locus, suggesting that the reduction in DNA methylation in Th2 conditions may help stabilize rather than initiate high-level Th2 cytokine expression. By contrast, the Flavell group has recently reported that one specific CpG in the Th2 locus control region (lying in the 3′ portion of the RAD50 gene) undergoes complete demethylation within 48 h of activation in Th2 conditions in a manner suggesting active rather than passive DNA demethylation [138]. These results suggest that focused DNA demethylation in the locus control region may play a role in the rapid increase in IL-4 expression in Th2 conditions, while passive demethylation in the locus may progressively contribute to sustained high-level Th2 cytokine expression. Consistent with the latter possibility, in long-term murine Th2 clones, different levels of demethylation at IL4CNS1 and the second intron are associated with quantitatively different levels of IL-4 expression [135]. In human Th2 cell populations polarized from adult $CD45RA^+$ cells, changes in DNA methylation at IL4CNS1, promoter and intron 2 are not pronounced, but a focus of demethylation around the IL-13 promoter and first exon has been mapped [137].

These studies implicate DNA methylation in the regulation of the Th2 cytokines in $CD4^+$ T cells, but in $CD8^+$ T cells this mechanism may play an even more profound role [51,64,99]. While wild-type naïve $CD4^+$ T cells express low but detectable amounts of IL-4, IL-13, and IL-5 mRNA after activation in non-polarizing conditions, naïve $CD8^+$ T cells do not express these cytokines in a cell autonomous manner (i.e., unless they are cultured in Th2/Tc2 conditions). In

sharp contrast, in T cells from conditional Dnmt1$^{-/-}$ mice, induction of these cytokines is markedly higher than in control T cells, but with a distinctive lineage bias: ∼10-fold in peripheral CD4^{+} T cells and ∼1000-fold in peripheral CD8^{+} T cells. As a result, expression of these Th2 cytokines by Dnmt1$^{-/-}$ CD8^{+} and CD4^{+} T cells was comparable and greater than expression by control CD4^{+} T cells. Analysis of CpG methylation, histone modifications and transcription factor expression suggest that the increased expression of IL-4, IL-13 and IL-5 resulted from DNA demethylation and a secondary increase in transcriptionally favorable histone modifications in the IL-4/IL-13 locus [51,99]. Loss of DNA methylation does not override other factors regulating Th2 cytokine expression, but rather appears to act in concert with them. When Dnmt1$^{-/-}$ CD4^{+} and CD8^{+} T cells are exposed to Th2/Tc2 polarizing conditions, IL-4, IL-5 and IL-13 increase further and Tc2 cells produce at least as much as do Th2 cells, while in Th1/Tc1 conditions expression declines but remains substantially greater than by control T cells cultured in non-polarizing or Th1/Tc1 conditions [99]. T cells from MBD2-deficient mice, like Dnmt1$^{-/-}$ T cells, exhibit enhanced expression of IL-4 in Th2 and even in Th1 conditions [108]. Together, findings in conditional Dnmt1$^{-/-}$ and MBD2$^{-/-}$ T cells support a model in which DNA methylation, methylated DNA binding proteins, post-translational histone modifications and the abundance of Th2 transcription factors collaborate to control appropriate patterns of IL-4 and Th2 cytokine expression (Fig. 4).

4.2.4. Cytolytic function

DNA methylation appears to repress expression of the gene encoding perforin in human T and NK cells, based on studies in which T cells are treated with 5-azacytidine. Treatment is associated with DNA demethylation in a region upstream of the core promoter, which is demethylated constitutively in NK cells [139]. By contrast, there is little or no increase in expression of perforin or granzyme B in naïve Dnmt1$^{-/-}$ CD4^{+} or CD8^{+} T cells directly ex vivo or 3 days after activation in vitro [99]. Whether this discordance is due to differences between human and murine T cells or technical aspects of these studies is unclear. NK cells and some CD8^{+} T cells express activating and inhibitory receptors of the Ly49 (mouse) and KIR (human) families in a clonally restricted and often monoallelic manner. Expression has been correlated with DNA demethylation of CpG near the transcription start site and treatment of cells with 5-azacytidine induces demethylation and ectopic expression of unexpressed receptors [140,141].

4.2.5. T cell effector function lineage commitment

Naïve CD4^{+} and CD8^{+} T lymphocytes emigrate from the thymus to secondary lymphoid organs with distinct differences in effector function potential. Naïve CD4^{+} T cells can differentiate into Th1 or Th2 cells, under the control of the 'master regulator' transcription factors T-bet and GATA-3, respectively. By contrast, CD8^{+} T cells emerge from the thymus pre-committed to differentiate into cells that produce

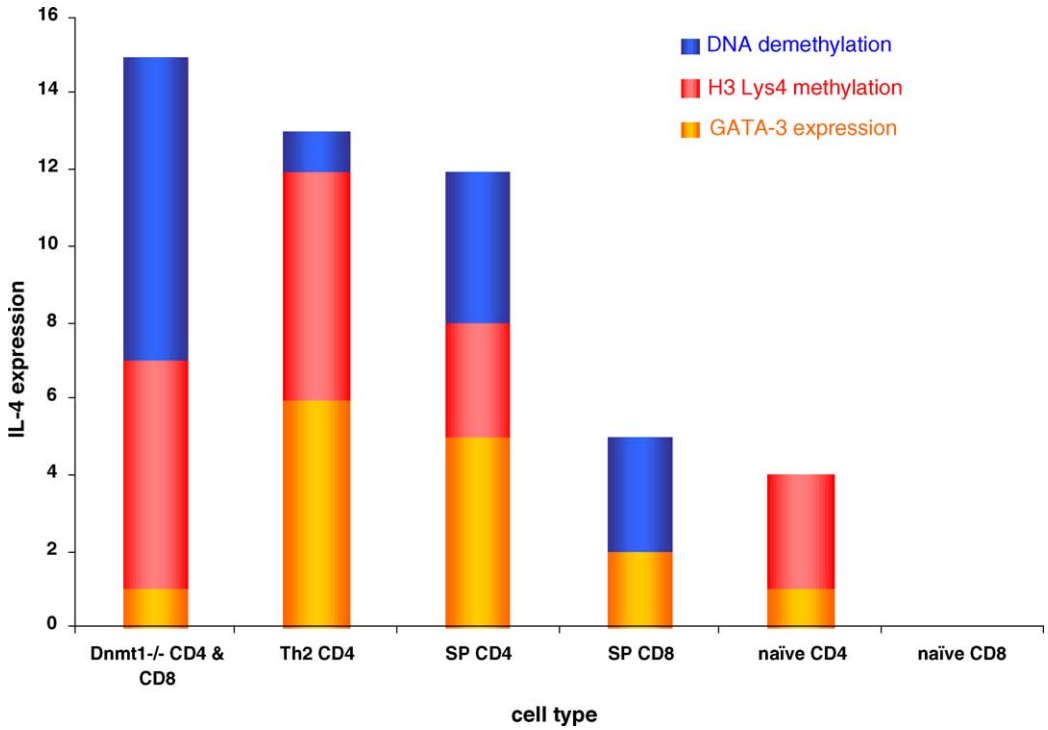

Fig. 4. The cumulative effect of GATA-3, DNA methylation and H3 Lys4 methylation on IL-4 expression. A model based on data from references [51] and [99].

IFN-γ, and the lytic effectors perforin and granzyme B, but can be induced to differentiate into Tc2 cells that produce IL-4, IL-13 and IL-5 by exposure to exogenous IL-4 [142,143]. Also, while T-bet is essential for the expression of IFN-γ and the Th1 fate in $CD4^+$ T cells, this is not true in $CD8^+$ T cells, which also express the T-bet paralog eomesodermin.

The findings described in the previous sections raise the following questions: when does epigenetic control of T cell effector function begin, when does effector lineage begin to be independent of the relevant 'master regulator' of that fate, when (if ever) is lineage commitment indelible so that the lineage-specific 'master regulator' is no longer required to sustain commitment, and what is the role of DNA methylation in the initiation and maintenance of commitment? Though studies in this area are incomplete, some clues are emerging.

In exploring the origin of the bias against Th2 cytokine expression by $CD8^+$ T cells, Makar et al. tracked the bias back to the SP thymocyte stage. While activated $CD4^+$ and $CD8^+$ SP thymocytes both express IL-4 mRNA, SP CD8 thymocytes express ∼100-fold less. This difference is paralleled by lower levels of GATA-3, paucity of transcriptionally favorable histone modifications, and modestly higher levels of DNA methylation in the IL-4/IL-13 locus in $CD8^+$ SP thymocytes [51]. DNA methylation increases and IL-4 and GATA-3 expression decline as cells mature from SP thymocytes to naïve T cells, in concert with recruitment of Dnmt1 throughout the locus in $CD4^+$ and $CD8^+$ SP thymocytes and Dnmt3b at IL4CNS1 in $CD4^+$ SP thymocytes. Differences in DNA methylation between $CD4^+$ and $CD8^+$ SP thymocytes are no longer evident in naïve $CD4^+$ and $CD8^+$ T cells, and so could not account for the fact that naïve $CD4^+$ but not $CD8^+$ T cells are able to produce small amounts of IL-4 in response to activation. Rather, residual IL-4 expression is associated with continued expression of GATA-3 by naïve $CD4^+$ but not $CD8^+$ T cells [51], and greater expression of the repressor of GATA3 (ROG) protein by $CD8^+$ than by $CD4^+$ T cells [144]. GATA-3 can competitively displace MBD2 from regulatory regions of the IL-4 gene (whether this is a direct or indirect effect is unknown), so GATA-3 may be the "pioneer factor" that opens the locus to epigenetic remodeling machinery [108]. However, there are other GATA-3-regulating factors such as friend-of-GATA-1 (FOG), mel-18, Gfi-1 and Notch that may also be involved in this pathway and have not yet been studied in $CD8^+$ T cells [145–148]. Another key may be intergenic transcription which, for the human IL-4/IL-13 locus, may also exhibit precocious expression in the thymus [149]. It will be interesting to see what signals and *trans* factors control intergenic transcription, what *cis* factors in the IL-4/IL-13 locus serve as initiation points, and what epigenetic machinery is most affected by active but low-level transcription. A fourth additional possibility is the presence of specific repressor or silencer elements with lineage-specific activity. Such elements may exist downstream of the IL-4 stop codon in the 3′ untranslated region [150] or in DNase hypersensitivity site IV [151], but their activities in $CD4^+$ versus $CD8^+$ T cells have not yet been examined.

Although differences in DNA methylation in the IL-4/IL-13 locus do not appear to account for differences in expression between naïve $CD4^+$ and $CD8^+$ T cells, the studies with $Dnmt1^{-/-}$ T cells indicate that DNA methylation is necessary to repress IL-4, IL-13 and IL-5 expression in both lineages and in its absence both lineages express IL-4 in high amounts [99]. These findings are consistent with those in $MBD2^{-/-}$ T cells (although only $MBD2^{-/-}$ $CD4^+$ T cells have been studied). This suggests that DNA methylation, perhaps working through MBD2-mediated recruitment of histone and chromatin modifying complexes, is likely to be essential for stable Th1 and $CD8^+$ effector lineage commitment, but this remains to be shown in vivo. Moreover, it is also unknown whether these processes alone would be sufficient to maintain commitment in the absence of the 'master regulators', T-bet and eomesodermin.

Although the IFN-γ gene has not yet been studied in detail in thymocytes, some [152] but not other [51] studies have found higher IFN-γ expression potential in $CD8^+$ compared to $CD4^+$ SP thymocytes and, levels of H3K4-Me at the IFN-γ promoter are higher in resting (but not activated) single-positive $CD8^+$ thymocytes [51]. Nevertheless, epigenetic remodeling of the IFN-γ gene clearly continues in the periphery during Th1/Th2 and Tc1/Tc2 polarization in response to environmental influences [105,114,117,119,122,153]. Cells differentiated under standard Th1 conditions in vitro (IL-12 plus anti-IL-4) for one week have an unstable Th1 phenotype, but cells differentiated for 3 or more weeks cannot be induced to produce Th2 cytokines when switched from Th1 to Th2 conditions [154], consistent with a model of progressive commitment to the Th1 phenotype over time [1,155]. Stable commitment to IFN-γ expression requires the collaboration of T-bet with another transcription factor Hlx, which itself is induced by T-bet. This committed state correlates not with maximal expression of IFN-γ or chromatin remodeling in the IFN-γ promoter and introns, both of which occur as early as one week. Rather, stability correlates most closely with extended DNA demethylation in the IFN-γ gene. Also, recent reports suggest that stable repression of IL-4 in Th1 cells correlates with spreading of DNA methylation to involve the CNS2/HSV region containing the 3′ IL-4 enhancer [1,136]. Together, these observations implicate DNA methylation in long-term maintenance but not initiation of Th1 polarization, DNA demethylation in the IFN-γ gene perhaps rendering IFN-γ expression T-bet-independent and methylation in the IL-4/IL-13 locus stably repressing IL-4 and the Th2 phenotype. However, the extent to which these associations can be generalized, and whether the changes in DNA methylation are cause or effect has not been tested. Moreover, these studies all relate to cells cultured in vitro under defined, strongly polarizing conditions. There is some evidence for epigenetic stability in the IFN-γ gene in mouse and human T cells with "memory" phenotypes [106,120]. Grogan et al. have shown that the IL-4/IL-13 locus in Th1 cells taken from *Leishmania major*-infected C57BL/6 mice has the repressive H3K9-Me mark and becomes repositioned to heterochromatin, suggest-

ing stable repression in this context has at least some of the characteristics found with Th1 cells generated in vitro [156]. However, further analyses and mechanistic studies of bona fide memory T cells during antigen-specific restimulation and attempted repolarization in vivo are needed.

5. Conclusions and future directions

The above literature suggests that DNA methylation, MeCpG-binding proteins, histone modification, transcription factors and chromatin remodeling complexes work cooperatively to regulate important inherited events in the T cell compartment of the immune system. These range from some of the earliest events in developing thymocytes to possibly some of the latest events in memory T cells. Since alterations in TCR repertoires, IFN-γ expression and IL-4 expression are strongly implicated in autoimmune and allergic diseases, it is not inconceivable that some diseases may possess an underlying "epigenetic immunopathology". If epigenetic lesions are fundamentally plastic and reversible, then "epigenetic therapy" is a plausible option and is already being considered in areas such as cancer [2].

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