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DNA Methylation in T-Cell Development and Differentiation

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Abstract

T lymphocytes undergo carefully orchestrated programming during development in the thymus and subsequently during differentiation in the periphery. This intricate specification allows for cell-type and context-specific transcriptional programs that regulate immune responses to infection and malignancy. Epigenetic changes, including histone modifications and covalent modification of DNA itself through DNA methylation, are now recognized to play a critical role in these cell-fate decisions. DNA methylation is mediated primarily by the actions of the DNA methyltransferase (DNMT) and ten-eleven-translocation (TET) families of epigenetic enzymes. In this review, we discuss the role of DNA methylation and its enzymatic regulators in directing the development and differentiation of $CD4^+$ and $CD8^+$ T-cells.

Keywords

epigenetics; lymphocyte; thymocyte development; DNMT1; DNMT3; TET

I. INTRODUCTION

T lymphocytes are a critical arm of the adaptive immune system that requires complex, tightly controlled orchestration of gene expression during developmental processes and immune responses. As T-cells develop in the thymus, they undergo a series of coordinated cellular decisions that ultimately result in commitment to a stable lineage. Then, once mature and upon egress from the thymus, naïve (antigen-inexperienced) T-cells patrol the periphery until they are activated by cognate antigen and undergo differentiation into discrete functional subsets. Activated T-cells have the potential to acquire many cell fates as they differentiate. At all stages, they must integrate a multitude of extracellular signals, including cytokines, chemokines, and metabolites that promote cellular programming. These

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developmental and cell-fate choices are regulated not only by transcription factor expression but also by epigenetic regulators that alter the epigenomic landscape. Epigenetic alterations include posttranslational modifications of histones and covalent modification of DNA through DNA methylation. These changes can alter the chromatin structure and accessibility at cis-regulatory elements, such as promoter or enhancer regions, to promote or repress gene transcription. Although DNA methylation has long been considered relatively stable, recent discovery of the ten-eleven translocation (TET) family of proteins identified the formation of oxidized methylcytosine derivatives and an active process of DNA demethylation. These processes are critical in the development, differentiation, and cell-fate stability of T lymphocytes. In this review, we specifically focus on how DNA methylation and its enzymatic regulators direct T-cell development and differentiation.

II. DNA METHYLATION

DNA methylation covalently modifies DNA through the methylation of the fifth carbon of a cytosine base (5-methylcytosine; 5mC), which in mammals occurs primarily in the context of CpG dinucleotides.[1,2] This process can occur through *de novo* DNA methylation or maintenance of DNA methylation during replication. In mammals, the two major catalytically active *de novo* DNA methyltransferases are DNMT3A and DNMT3B.[3,4] These enzymes transfer the methyl group of the coenzyme S-adenosyl-L-Methionine (SAM) to the cytosine residue of DNA[5,6] and bind equally unmethylated cytosines or hemimethylated cytosines.[3,7] DNMT1 is responsible for the maintenance of DNA methylation during DNA replication. In contrast to DNMT3A/B, DNMT1 preferentially binds hemimethylated DNA.[8–10] In resting cells, DNMT1 is maintained in an autoinhibitory conformation.[11–13] During replication, the E3 ubiquitin ligase UHRF1 recruits DNMT1 to hemimethylated CpG dinucleotides at replication forks,[14,15] allowing DNMT1 to methylate the daughter DNA strand[13] and thereby maintaining DNA methylation during cellular replication.

The DNMT proteins contain an N-terminal regulatory domain and a C-terminal catalytic domain (Fig. 1A). DNMT1 is by far the largest protein of the DNMT family and contains numerous domains including the N-terminal independently folded domain (NTD), replication foci-targeting sequence domain (RFTS), a CXXC motif, two bromo-adjacent homology domains (BAHs), and the catalytic domain. The NTD binds multiple proteins that can serve to regulate DNMT1 function. One of these is PCNA, a required factor for cellular replication, which binds in the NTD of DNMT1 and helps maintain the methylation of daughter DNA.[16] The RFTS domain mediates DNMT1 localization to replication forks in late S-phase[17] in a UHRF1-dependent manner and is required for replication-dependent maintenance of DNA methylation.[14,15] The CXXC domain is a conserved zinc-finger domain that can bind unmethylated CpG-containing DNA; however, two reports[10,18] have opposing findings regarding the requirement of the CXXC domain for DNMT1 enzymatic activity; thus, further work is needed to clarify its role in DNMT1 function. The function of the two BAH domains remain to be elucidated. All DNMT enzymes have a C-terminal catalytic domain that contains 10 motifs characteristic of DNA-(cytosine C5)-methyltransferases. The DNMT family uses SAM as a methyl group donor and a base-flipping mechanism that rotates the target base into the catalytic pocket.[19]

DNMT3A and DNMT3B have similar domain structures: each contains a PWWP, an ADD, (Atrx-DNMT3-DNMT3L), and a C-terminal catalytic domain. The PWWP domain binds DNA[20,21] and histone tails[22] to tether DNMT3A/B to chromatin to allow for DNA methylation.[23,24] Interestingly, a point mutation in the PWWP domain of DNMT3B was identified as the cause of the immunodeficiency, centrometric instabilities and facial anomalies (ICF) syndrome in humans.[25] The ADD domain also interacts with histone tails, [26–28] as well as multiple other reported proteins (reviewed by Tajima et al.[29]). The ADD domain of DNMT3A is located such that its positioning inhibits access of DNA to the catalytic domain.[30] Binding of the N-terminal tail of histone H3 to the ADD domain alters the ADD domain positioning to allow DNA access to the catalytic domain, potentially enhancing DNMT3A enzymatic activity.

For many years, it was assumed that inhibition of DNMT1 activity was the primary mechanism through which DNA demethylation occurred. However, identification of the TET family of enzymes shed light on the process of active DNA demethylation. TET1 was originally identified as a fusion partner with the MLL protein in a t(10;11) translocation in acute myeloid leukemia.[31,32] Three TET family members were subsequently identified (TET1–3) through homology to the trypanosome base J-binding proteins (JBP1 and JBP2), which hydrolyze the methyl group of thymine.[33,34]

All TET family members contain two conserved domains in the C-terminus, which together form the catalytic domain of TET proteins: a cysteine-rich region and a double-stranded β-helix (DSβH) fold domain interrupted by a low complexity nonconserved region (Fig. 1B). Contained within the DSβH domain are key residues that mediate α-ketoglutarate and Fe (II) binding, cofactors required for TET enzymatic function.[33,34] TET1 and TET3 contain an N-terminal CXXC domain, which can bind unmethylated CpG dinucleotides; however, an ancestral chromosomal inversion led to the loss of the CXXC domain in the TET2 locus, and in mammals it is encoded by a distinct gene *IDAX (CXXC4)*.[35] How the structural differences in TET family members may influence their function is currently unknown. IDAX downregulated TET2 protein expression through caspase-dependent degradation in murine embryonic stem cells and a human monocytic cell line[35]; however, IDAX is not expressed in naïve or recently activated murine T lymphocytes[36] and whether such a mechanism exists in T-cells to regulate TET2 expression is currently unclear.

TET proteins mediate DNA demethylation through the sequential oxidation of 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC)[33,37–39] (Fig. 2). These oxi-methylcytosine bases can promote passive DNA demethylation during replication as the DNMT1/UHRF1 complex prefers a hemimethylated substrate over a hemihydroxymethylated or unmethylated DNA base[40–42]; thus, their presence will inhibit maintenance of DNA methylation. Additionally, both 5fC and 5caC can be converted to an unmodified cytosine through the action of thymine DNA glycosylase (TDG) and the base excision repair pathway.[37,43–45]

Despite similar amounts of 5mC across different cell types, 5hmC levels have been shown to vary; neuronal cells contain the most, upward of nearly 40% of 5hmC in postmitotic Purkinje neuronal cells.[33,46–48] Due to this accumulation, 5hmC is frequently viewed as a

relatively stable epigenetic mark, and 5hmC, and possibly other oxi-methylcytosine intermediates, may serve as distinct epigenetic marks rather than simply intermediates in the DNA demethylation process. Several groups have identified proteins that selectively bind 5hmC-containing DNA,[49,50] and 5hmC may prevent binding of some 5mC readers[40,51,52]; however, the functional consequences of 5hmC as a distinct epigenetic mark remain unclear. In T-cells, 5hmC is enriched at cell-type specific enhancer regions and in the gene bodies of actively transcribed genes,[53] suggesting that regulation of this epigenetic mark may have functional consequences for gene expression. Although DNA methylation at promoter regions is widely accepted as a repressive mark for gene transcription, how the presence of 5mC or its oxidized intermediates at other cis-regulatory regions, such as enhancers, affects gene expression is not yet fully understood.

TET2 and DNMT3A loss-of-function mutations occur at high frequency in hematologic malignancies. Originally identified in human myeloid neoplasms,[54–58] later studies in T-cell lymphomas found that 50%–70% of certain T-cell lymphomas[59–62] contained TET2 and/or DNMT3A mutations, suggesting that these enzymes play a critical role in immune cell development and homeostasis.

III. T-CELL DEVELOPMENT

Thymopoiesis is a tightly regulated process involving multiple developmental stages that ultimately results in mature T lymphocytes. Common lymphoid progenitor (CLP)-derived early thymic progenitors (ETPs) seed the thymus from the bone marrow. Developing thymocytes then progress through multiple stages defined by their expression of CD4 and CD8 coreceptors: (1) double-negative (DN; $CD4^{-}CD8^{-}$), (2) double-positive (DP; $CD4^{+}CD8^{+}$), and (3) CD4 single-positive (CD4SP; $CD4^{+}CD8^{-}$) or CD8 single-positive (CD8SP; $CD4^{-}CD8^{+}$). DN thymocytes are subsequently subdivided into four groups according to surface expression of CD44 and CD25: DN1 ($CD44^{+}CD25^{-}$), DN2 ($CD44^{+}CD25^{+}$), DN3 ($CD44^{-}CD25^{+}$), and DN4 ($CD44^{-}CD25^{-}$). As developing thymocytes transit through these stages, they pass through several developmental checkpoints. T lineage commitment, associated with concomitant loss of alternative lineage potential, occurs stepwise through the DN1 and DN2 stages (see review by Yui and Rothenberg[63]). In the DN3 stage, thymocytes undergo β-selection, which requires αβ T-cell precursors to signal through a functional pre–T-cell receptor (TCR) consisting of a productively rearranged TCRβ and invariant pre-Tα chain. Following β selection, the developing thymocytes initiate CD4 and CD8 transcription and upregulate TCRα expression to transition into the DP stage. At this point, thymocytes must navigate positive and negative selection, during which time DP cells are rescued from programmed cell death if they have the ability to interact with self-MHC presenting self-peptides (positive selection) and to avoid TCR-induced cell death if they react too avidly with self-peptide (negative selection) (reviewed in Stritesky et al.[64]). The small proportion of DP thymocytes that successfully survive these checkpoints downregulate CD4 or CD8 coreceptor expression to become CD8SP or CD4SP cells, respectively. Clearly, a multitude of factors, including transcription factors, chemokines, and cytokines, regulate these developmental processes, and more recent studies have shed light on how DNA methylation contributes to thymocyte development.

Genome-wide DNA methylation studies in murine hematopoietic lineage commitment revealed differential methylation at multiple stages of thymocyte commitment,[65] suggesting that DNA methylation is dynamically regulated during T-cell development. At the CLP to DN1 stage, there were more differentially methylated regions with gain-of-methylation versus loss-of-methylation, potentially suggesting that *de novo* methyltransferase activity may play a key role at these stages. Overall, this study noted a skewing toward greater genomic methylation in lymphoid compared to myeloid progenitors. Consistent with this observation, the addition of 5-azacytidine, a DNA methyltransferase inhibitor, to the OP9-DL1 stromal co-culture system (an *in vitro* system that promotes both lymphoid and myeloid commitment from progenitors) led to an increase in myeloid commitment from multipotent progenitors (MPPs), CLPs, DN1, and DN2 cells at the expense of lymphoid commitment. These data indicate that DNA methylation plays a functional role in determining T lineage commitment and are consistent with the findings that hypomorphic DNMT1 alleles can sustain myeloid but not lymphoid commitment.[66] Examination of genome-wide 5hmC in later stages of thymocyte development (DP, CD4SP, and CD8SP) revealed enrichment in thymus-specific active enhancers (defined by co-occurring H3K27ac and H3K4me1 marks) compared to embryonic stem cell and murine embryonic fibroblast-specific enhancers.[53] Additionally, this study noted dynamic changes in 5hmC distribution during differentiation stages and a strong correlation between 5hmC intragenic enrichment and gene expression. Together, these data suggest that the TET enzymes may play a role in thymopoiesis. During lineage commitment, the CD8 and CD4 coreceptors facilitate TCR signaling during selection as coreceptors for MHC class I and class II, respectively. Positive selection of cells with TCRs specific for MHC class I develop into CD8SP cells, and cells with MHC class II-specific TCRs develop into CD4SP. The expressions of the CD4 and CD8 coreceptors have been shown to be epigenetically regulated (see review by Issuree et al.[67]), and DNA methylation mediated via the DNMT family was identified as a mechanism for heritable silencing of the *CD4* locus in mature $CD8^+$ T-cells[68] and TET1/TET3-mediated methylation as controlling enhancers that regulate CD4 expression in mature $CD4^+$ T-cells.[69]

During human αβ T-cell development, global DNA methylation reprogramming occurs during several key developmental checkpoints: T-cell lineage commitment, β-selection, TCRαβ expression, and positive selection with both gain and loss of DNA methylation.[70] Notably, more differentially methylated regions underwent demethylation with fewer undergoing *de novo* methylation at all differentiation steps except for initial T-cell lineage commitment, in line with the gain-of-methylation seen in the CLP and DN1 stages in murine cells.[65] These methylation changes remained mostly stable at later differentiation stages; however, notable exceptions to this finding included the *CD8a*, *PTCRA*, and *Rag1* loci. Changes in DNA methylation tended to occur at genomic loci associated with T-cell differentiation or T-cell receptor function based on gene ontology analysis, supporting a regulatory role for DNA methylation during thymocyte development. When the same researchers examined the relationship between differentially methylated regions and differentially expressed genes, ~85% of the demethylation events correlated with an increase in gene expression during concurrent differentiation step. This finding may reflect the fact that promoter methylation correlates well with gene repression.

To begin to understand how these global changes in DNA methylation occur during T-cell development, several studies have focused on the enzymes responsible for regulating the methylated state of DNA (Table 1; Fig. 3). Early studies examined the role of DNMT family members in T-cell development. Inducible loss of DNMT1 just prior to the DN to DP transition resulted in a profound loss of DP and SP thymocytes due to increased apoptosis.[71] Once past the DP transition, loss of DNMT1 (mediated by CD4Cre) resulted in essentially normal T-cell development with a slight reduction in $CD44^{hi}$ populations of peripheral $CD4^+$ and $CD8^+$ T-cells.[71] These data support a critical role for DNMT1 in early T-cell development.

A role for *de novo* DNA methylation by DNMT3A in thymopoiesis has also been identified. DNMT3A knockout mice develop normally but become stunted and die by approximately 4 weeks of age.[4] Evaluation of thymocyte development in these neonates revealed a marked decrease in thymic cellularity with a partial DN to DP block in the DNMT3A-deficient mice and splenic hypocellularity.[72] However, the stunted phenotype in these mice makes it difficult to draw clear conclusions regarding the role of DNMT3A in T-cell development, thus making targeted knockout mice essential for the study of DNMT3A in thymopoiesis. Inducible loss of DNMT3A in hematopoietic stem cell (HSC) progenitors can lead to an accumulation of DN2 thymocytes but only after secondary transplantation into recipient hosts and only in ~40% of these recipients.[73] The partial penetrance of this effect and fact that it was not seen in primary transplant recipients suggest that other selection events may need to occur for DNMT3A loss to significantly affect thymocyte development. When DN2 expansion did occur, it was associated with downregulation of Nur77 and a decrease in apoptosis. In the same study, DNMT3A loss was shown to cooperate with Notch gain-of-function mutants to promote T-cell acute lymphoblastic leukemia (T-ALL). Importantly, using different Cre drivers, the authors determined that DNMT3A loss had to occur at the ETP stage to cooperate with Notch gain-of-function driving leukemic transformation. The resulting experimental DNMT3A-deficient T-ALLs had a gene expression signature that resembled human ETP-ALL, which has been noted to have a high rate of DNMT3A mutations.[74] Together, these data suggest that DNMT3A loss in early thymic progenitors may prime developing thymocytes for cellular transformation, but it does not play a critical role in early thymopoiesis under most physiologic conditions. The role of DNMT3B has not been assessed in developing T-cells, though it is expressed.[75]

The TET family of enzymes also play a role in T-cell development (Table 1). Combined loss of TET2 and TET3 (either $Tet2^{-/-}TET3^{fl/fl}CD4Cre^+$ or $TET2^{fl/fl}TET3^{fl/fl}CD4Cre^+$) led to a striking lymphoproliferation of invariant natural killer T (iNKT) cells, ultimately resulting in death by 8 weeks of age.[76] iNKT cells are a rare subset of innate-like lymphocytes that develop in the thymus, express a semi-invariant αβ TCR that recognizes glycolipid antigen and have a mature effector phenotype. Similar to $CD4^+$ T-cells, they can be subdivided into functional subsets (NKT1, NKT2, and NKT17) that express unique transcription factors and cytokine profiles (IFNγ, IL-4, and IL-17, respectively).[77] In TET2/TET3-deficient mice, the iNKT cell expansion occurred in the thymus, presumptively during T-cell development. The expanded TET2/TET3-deficient iNKT cells were of the NKT17 phenotype, with RORγt expression and IL-17 production, in contrast to TET2 loss in conventional $CD4^+$ T-cells, which results in a decrease in T_H17 differentiation compared to wild-type.[78] Although

5hmC levels were substantially reduced in the double-knockout iNKT cells, they were not abolished, suggesting that TET1 was active in these cells. A key experiment in which the TET2/TET3-deficient iNKT cells were transferred into immunocompetent wild-type or CD1d-deficient hosts (which lack the MHC that presents glycolipid antigen for iNKT cells) demonstrated the following: (1) The lethal lymphoproliferative disorder was transferable to wild-type hosts, thus supporting that these were malignant cells and wild-type Tregs could not suppress their proliferation. (2) The CD1d-deficient recipients remained healthy demonstrating that antigen was required for expansion. The critical finding that TET2/TET3 restricted an antigen-driven process suggests that the TET proteins may be regulated and function downstream of antigen activation. No studies have been published to date on the role of TET1 in thymocyte development.

IV. CD4+ T-CELL DIFFERENTIATION

$CD4^+$ T-cells orchestrate immune responses through provision of critical help to $CD8^+$ T-cells, B cells, or innate immune cells, typically through cytokine production and/or cell-to-cell interactions. Upon antigen recognition, naïve $CD4^+$ T-cells are activated, proliferate, and differentiate into T helper (T_H) subsets with distinct functional capabilities in response to environmental stimuli. These effector T_H subsets are classically defined based on expression of 'master' transcription factors and their unique cytokine production. T_H1 cells express T-bet and produce IFNγ. T_H2 cells express GATA-3 and produce IL-4. ROR-γt is critical for T_H17 differentiation and IL-17A/IL-17F production. T follicular helper (T_{FH}) cells are defined by Bcl-6 expression and IL-21 production. Regulatory T-cells (Tregs) express Foxp3 and produce IL-10. Although the field has traditionally categorized these T_H subsets based on transcriptional regulators and prototypical cytokine production, it has been increasingly recognized that they also undergo extensive epigenomic re-programming during differentiation, including changes in DNA methylation.

A. T_H1/T_H2 Differentiation

One of the first observations suggesting that DNA methylation plays a critical role in $CD4^+$ T-cell differentiation came from early studies of T_H1 versus T_H2 differentiation. Several groups noted that the *ifng* genomic locus is hypomethylated in naïve T-cells and the *il4* locus is hypermethylated. Under *in vitro* T_H1-polarizing conditions, this pattern is maintained; however, under T_H2 conditions, the *ifng* locus becomes hypermethylated and the *il4* locus is hypomethylated in both human and murine $CD4^+$ T-cells.[79–87] Moreover, treatment of low IL-4–expressing T_H2 clones with 5-azacytidine, a hypomethylating agent, resulted in increased IL-4 expression, suggesting that the degree of DNA methylation can regulate the magnitude of cytokine production in differentiated $CD4^+$ cells.[88] More recently, genome-wide DNA methylation/hydroxymethylation studies demonstrated that murine and human $CD4^+$ T-cells gain 5hmC primarily at intragenic sites during *in vitro* T-helper differentiation.[53,89] In murine cells, gain of 5hmC occurred at lineage-specific gene bodies and correlated with increased expression: T_H2 cells had increased 5hmC and decreased 5mC at *Gata3* and *il4* loci whereas T_H1 cells displayed increased 5hmC at *ifng* and *Tbx21* loci.[53,78] Together, these data suggest that dynamic DNA methylation remodeling occurs at key lineage-specific

loci, supporting a role for DNA methylation in the regulation of T_H1 versus T_H2 differentiation.

Whether DNA methylation at lineage-specific loci serves as a mechanism to establish T_H1 and T_H2 cell fates or is a consequence of differentiation was addressed with mechanistic studies examining the loss of DNA methylation regulators. The DNMT family has been implicated in various aspect of T_H1 versus T_H2 differentiation. DNMT1 loss (mediated by CD4Cre) led to decreased peripheral T-cell proliferation and enhanced expression of IFNγ, IL-2, IL-3, and IL-4 in activated $CD4^+$ (and $CD8^+$) T-cells,[71] suggesting that DNMT1 serves to repress cytokine production. Under T_H2 polarizing conditions, DNMT1 undergoes dissociation (or decreased recruitment) from the *il4* locus, permitting demethylation of the locus and increased expression of IL-4.[90] Together, these data indicate that DNMT1 loss at effector cytokine loci is a critical step to allowing IL-4 expression during T_H2 differentiation.

In contrast, *de novo* methylation by DNMT3A does not affect initial T_H1 versus T_H2 differentiation but does alter cell fate plasticity. Powell and colleagues demonstrated that DNMT3A-deficient naïve $CD4^+$ T-cells develop a population of cells capable of coexpressing IL-4 and IFNγ when they are activated through the TCR in neutral conditions *in vitro*. These changes were associated with demethylation of both loci. However, under polarizing conditions, loss of DNMT3A did not significantly alter T_H1 or T_H2 differentiation and no double-producing cytokine population was noted.[72] DNMT3A is recruited to the *ifng* promoter in murine T_H2, T_H17 and iTreg cells but not in T_H1 cells,[91] and the *ifng* locus is unmethylated in DNMT3A-deficient T_H2 and T_H17 cells.[72, 91] However, lack of DNMT3A in these cells was not sufficient to allow IFNγ expression under non-T_H1 conditions, suggesting that methylation alone was not enough to suppress IFNγ expression under these conditions. When DNMT3A-deficient T_H2 or T_H17 cells were re-cultured under T_H1 biasing conditions,[72,91] there was a consistent increase in IFNγ expression compared to the wild-type, suggesting that *de novo* methylation by DNMT3A restricts T_H plasticity.

TET family members have also been implicated in directing T_H1 versus T_H2 differentiation. Deletion of TET2, driven by CD2Cre, resulted in increased methylation of an IFNγ enhancer with decreased IFNγ expression following *in vitro* T_H1 polarization, with no effect on *Tbx21* transcription (encoding T-bet); however, TET2 loss did not lead to alteration in T_H2 differentiation.[78] Using chromatin immunoprecipitation, TET2 association and 5hmC was diminished on the *ifng* locus in T-bet deficient cells,[78] suggesting that T-bet may recruit TET2 to mediate hydroxymethylation at the *ifng* locus and promote IFNγ expression in T_H1 cells. Although TET1 regulation of T_H1 versus T_H2 cell fate has not been tested, overexpression of full-length TET1 (though not TET1 catalytic domain) in human $CD4^+$ T-cells under T_H1 polarizing conditions led to a downregulation of IFNγ,[89] suggesting a methylation-independent role of TET1 in regulating T_H1 cytokine production. The role of TET enzymes in limiting T-helper plasticity has not been evaluated.

B. Treg Differentiation

Regulatory T-cells (Tregs) are a subset of $CD4^+$ T-cells that limit inflammatory reactions. Expression of the Treg "master regulator" FoxP3 is essential for the development and function of Tregs and is controlled through three distinct intronic conserved noncoding sequences (CNS).[92–94] CNS2 is rich in CpG elements and particularly controlled by DNA methylation. Originally, it was identified as a highly conserved region that is methylated in conventional $CD4^+$ T-cells but demethylated in Tregs.[95,96] Although CNS2 is not required for the development of Tregs, it is required for FoxP3 stability and the maintenance of Treg cell fate.[92–94] CNS2 contains cis-regulatory elements that orchestrate the recruitment of transcription factors that promote or inhibit FoxP3 expression.[96–98] In addition to the FoxP3 locus, additional Treg specific demethylated regions (TSDRs) have been identified to control expression of Treg-associated genomic loci in thymically derived Tregs, including *Tnfsrf18*, *Ctla4*, and *Ikzf4* loci, in a FoxP3-independent manner, which occur during development and are not found in induced Tregs (i.e., those that arise in the peripheral lymphoid organs from conventional $CD4^+$ T-cells).[99] Thus, DNA methylation patterns control FoxP3 expression and promote the cell fate of thymically derived Tregs.

Given the critical role of DNA methylation in controlling FoxP3 expression and Treg cell fate, the naturally arising question is which epigenetic modifiers play a role in promoting and maintaining the methylation state of key cis-regulatory regions. Pharmacologic inhibition of DNMT activity with 5-azacytidine or genetic loss of DNMT1 promoted the expression of FoxP3 in thymic and peripheral FoxP3-negative $CD4^+$ T-cells upon TCR stimulation.[96,100,101] Together, these data support the notion that DNA methylation, likely through DNMT1, plays a key role in maintaining suppression of FoxP3 in non-Tregs. *In vivo*, male mice with a Treg-specific deletion of DNMT1 developed lethal autoimmunity by 3–4 weeks of age.[102] These mice had similar absolute numbers of thymic Tregs but decreased number of peripheral Tregs. The DNMT1-deficient Tregs had a marked loss of suppressive capacity *in vitro* and *in vivo*. Although methylation of the FoxP3 CNS2 was unchanged by DNMT1 loss in Tregs, global changes in DNA methylation were associated with loss of expression of several genes critical for Treg function and gain of inflammatory gene expression. Together, these studies support a role for DNMT1 in maintaining appropriate repression of FoxP3 expression in conventional $CD4^+$ T-cells and controlling a Treg gene expression program. Notably, DNMT3A was not required in Tregs *in vivo*,[102] suggesting that maintenance of DNA methylation rather than *de novo* methylation plays a more critical role in Treg development and function.

Active DNA demethylation through the TET family also serves to regulate Treg cell fate and stability. Initial experiments examining the role of TET in 'induced' Treg generation utilized ascorbic acid to promote TET enzymatic function. These studies demonstrated that ascorbic acid promoted FoxP3 expression and stability via demethylation of the *Foxp3* CNS2 enhancer in a TET2/TET3 dependent manner.[103,104] Mice with a Treg-specific deletion of TET2 and TET3 develop a lethal inflammatory disease, likely due to decreased long-term (but not short-term) suppressive function of thymic Tregs.[105] Mechanistically, Tregs from these mice had blunted demethylation at CNS2 of the *FoxP3* locus and other TSDRs, suggesting that TET2/TET3 are responsible for demethylation of FoxP3 CNS2 and TSDRs

of other Treg lineage genes. Additionally, TET2/TET3 loss led to an increase in 'ex-Tregs' (Tregs that had lost FoxP3 expression) consistent with the role of CNS2 FoxP3 and other TSDR methylation in maintaining FoxP3 stability and Treg function. It is likely that all TET family members cooperatively play a role in CNS2 demethylation and FoxP3 stability because TET1/2 double-deficient Tregs also have increased methylation and concomitant loss of Treg stability and function, resulting in increased inflammation and autoimmunity.[106] Together, these data point to unequivocal role for TET family members in regulating FoxP3 stability and Treg cell-fate maintenance.

In recent years, it has been increasingly appreciated that Tregs can undergo differentiation into specialized tissue-resident subsets that have distinct tissue-specific gene-expression profiles and functions (see review by Panduro et al.[107]). Recent work has explored the role of DNA methylation in tissue Treg programming. Genome-wide methylation analysis found that skin and adipose tissue Tregs have distinct DNA methylation profiles compared to Treg and conventional $CD4^+$ T-cells isolated from lymph nodes.[108] Interestingly, more differentially methylated regions were noted between tissue Tregs and lymph node Tregs than between lymph node Tregs and conventional $CD4^+$ T-cells. Many of the DMRs were shared between skin and fat Tregs, suggesting either a tissue Treg specific methylation program or effector Treg program. The role of the DNMT and TET families in regulating tissue-specific Treg methylation programming has not yet been explored.

C. T_H17 Differentiation

$CD4^+$ T helper cells that secrete IL-17 are termed T_H17 cells and are considered proinflammatory. During *in vitro* T_H17 differentiation, $CD4^+$ T-cells undergo dynamic changes in methylation states at lineage-associated loci.[78,109] Examining the *il17* locus, Wells and colleagues found lineage-specific DNA demethylation in T_H17 cells at the *il17a* and *il17f* loci. Specifically, demethylation was noted in a conserved enhancer region and at the promoters, and methylation-sensitive binding of STAT3 to the promoter enhanced transcriptional activity.[110] Stability of the T_H17 program seems to require DNMT3A, as this DNA methyltransferase was critical to suppressing IFNγ production in T_H17 cells repolarized under T_H1 conditions.[91]

During *in vitro* T_H17 differentiation, TET2 loss blunted IL-17A expression and was associated with decreased 5hmC deposition and RORγt binding to *il17* cis-regulatory regions. There was also a concomitant increase in IL-10 expression in these cells.[78] Similar to T_H1 cells, deletion of the T_H17 lineage-defining transcription factor *RORc* (encoding RORγt) led to decreased TET2 binding and 5hmC deposition at the *il17* locus, suggesting that TET2 recruitment may be mediated by lineage-specific transcriptional regulators.[78] Another group found that during *in vitro* T_H17 differentiation, T_H17 cells contain increased amounts of the metabolite 2-hydroxyglutarate (2-HG), which inhibits TET1/TET2-mediated demethylation of FoxP3 CNS2, leading to decreased FoxP3 expression and increased IL-17A production under T_H17 conditions.[111] Taken together, data suggest that TET2 activity can impact T_H17 cell differentiation by multiple mechanisms.

D. T_{FH} Differentiation

T_{FH} cells reside in the lymphoid follicle and interact with B cells to facilitate activation and germinal-center B-cell differentiation. At the transcriptional level, T_{FH} development is well characterized, but how epigenetic mechanisms facilitate commitment and maintenance of T_{FH} cells is not as well understood. Genome-wide methylation and hydroxymethylation profiling in naïve and T_{FH} $CD4^+$ T-cells, in combination with Bcl6 chromatin immunoprecipitation, exhibited a reduction in 5hmC at Bcl6 binding site in T_{FH}, but not naïve cells.[112] These differences were associated with decreased TET1 recruitment as assessed by chromatin immunoprecipitation, suggesting that Bcl6 may inhibit TET1 localization and activity at target genes; however, there was no assessment of the functional role of TET1 loss or other TET family members in T_{FH} differentiation.

A role for DNA methylation in T_{FH} cell fate is strongly suggested by the high frequency of TET2 and DNMT3a loss-of-function mutations in human T_{FH}-derived lymphomas.[59–62,113] Work in murine models has supported a role for TET2 loss being permissive but not sufficient for lymphomagenesis since expression of the dominant negative RhoA G17V mutation frequently found in these lymphomas in the setting of TET2 deficiency drove lymphomagenesis in these models.[114–116] Further work needs to be done to elucidate the contributions of TET and DNMT family members to T_{FH} differentiation.

E. $CD4^+$ T-Cell Memory

Given that DNA methylation can frequently be a heritable epigenetic mark, it is intriguing to examine how it may play a role in $CD4^+$ T-cell memory. Although we do not have a complete understanding of how DNA methylation regulates $CD4^+$ T-cell memory, comprehensive epigenomic and transcriptional profiling of human $CD4^+$ T-cell populations has demonstrated progressive loss of DNA methylation in heterochromatic regions across naïve, central memory, effector memory and terminally differentiated effector memory cells.[117] In this study, gene-specific differentially methylated regions were localized to enhancer or promoter regions and correlated with differences in the expression of memory-associated genes.[117] Murine studies indicate that differentiated $CD4^+$ memory cells (specifically T_H1- and T_{FH}- committed cells) can be distinguished based on differential methylation profiles.[118] Another study using TCR transgenic $CD4^+$ T-cells assessed genome-wide methylation of *in vitro* generated "memory" $CD4^+$ T-cells and found that differentially methylated regions were associated with enhancer activity of associated genes, rather than promoter activity.[119] Together, these data strongly support a role for DNA methylation globally directing $CD4^+$ memory differentiation; however, the role of individual TET or DNMT family members in directing or maintain $CD4^+$ T-cell memory remains unclear.

V. $CD8^+$ T-CELL DIFFERENTIATION

In response to microbes, naïve $CD8^+$ T-cells proliferate and differentiate into a heterogeneous pool of antigen-specific cells having divergent cell fates. Following pathogen clearance, most antigen-specific effector $CD8^+$ T-cells are terminally differentiated and undergo programmed cell death. A small subset persists to become long-lived memory cells, which are capable of rapidly responding to rechallenge. Elegant work by multiple groups has

identified different cell surface proteins that can be used to distinguish cells with differing memory potential. $CD8^+$ T-cells that are $CD127^{hi}$ and $KLRG1^{lo}$ preferentially differentiate into long-lived memory cells; whereas $CD127^{lo}$ $KLRG1^{hi}$ cells are primarily short-lived, terminally differentiated effector cells.[120–122] Memory $CD8^+$ T-cells have been further subdivided into $CD62L^+$ 'central memory' (T_{CM}) and $CD62L^-$ 'effector memory' (T_{EM}), with T_{CM} cells having the ability to self-renew and confer long-term immune protection.[123]

As murine and human $CD8^+$ T-cells differentiate, they undergo genome-wide epigenetic reprogramming, including changes in the DNA methylation landscape. Using methylated DNA immunoprecipitation, Scharer and colleagues demonstrated that antigen-specific $CD8^+$ T-cells undergo dynamic DNA remodeling during the naïve to effector $CD8^+$ T-cell transition following LCMV-Armstrong infection.[124] These researchers demonstrated that thymic enhancers (as marked by H3K4me1 and H3K27ac in ENCODE) underwent both methylation and demethylation, and they noted more differentially methylated regions occurred at active thymic enhancers. Additionally, DNA methylation at proximal promoter regions was inversely correlate with gene expression during the naïve to effector transition. The differentially methylated regions at both putative enhancer regions and gene promoters for differentially expressed genes were enriched for functional transcription factor motifs. Together, these data suggest that remodeling of DNA methylation plays a functional role in $CD8^+$ T-cell differentiation.

Building on these findings, Youngblood and colleagues utilized whole-genome bisulfite sequencing to examine the methylation programs of viral-specific naïve, short-lived effector $CD8^+$ T-cells and memory precursor $CD8^+$ T-cells at days 4.5 and 8 following LCMV-Armstrong infection. These studies revealed that memory precursor $CD8^+$ T-cells acquire a methylation program similar to terminally differentiated effector cells within the first several days of infection, supporting the hypothesis that memory precursor cells transition through an effector phase as they undergo memory differentiation.[125] Additionally, they found that DNMT3A-deficient $CD8^+$ T-cells underwent more rapid transition to memory cells compared to the wild-type. These data are consistent with an earlier report demonstrating DNMT3A loss promotes early $CD8^+$ T-cell memory development following viral infection. In this report, TCF-1 was proposed as a potential DNMT3a target responsible for directing wild-type $CD8^+$ T-cells toward an effector phenotype, and although knockdown of *TCF7* (which encodes TCF-1) did restore some effector T-cell differentiation in DNMT3A-deficient $CD8^+$ T-cells, the restoration was only partial, indicating that DNMT3A may target a wider array of genes that work together to direct $CD8^+$ effector T-cell differentiation.[126]

Whole-genome bisulfite sequencing of human naïve and memory $CD8^+$ T-cell subsets revealed that naïve, stem-cell memory, central memory, and effector memory subsets are epigenetically distinct, with a progressive decline in DNA methylation related to terminally differentiated state of cells (i.e., T_{EM} with the most demethylated regions).[127] To examine the stability of human memory $CD8^+$ T-cells *in vivo*, these researchers examined donor-derived memory $CD8^+$ T-cells before and after $CD45RA^+$ depleted haploidentical stem-cell transplants. Cells were isolated from the pre-transplant donor sample and then *in vivo* expanded donor-derived cells two months post transplant, isolated from the recipient. Methylation status at effector loci (e.g., IFNγ, perforin) was stable in the expanded memory

cells supporting the notion that transcriptionally permissive epigenetic programs are maintained at effector loci during *in vivo* homeostasis. Interestingly, most of the expanded memory cells had a T_{EM} phenotype with increased methylation at differentially methylated regions in loci encoding CD62L and CCR7 despite the pre-transfer samples having a mix of T_{EM} and T_{CM} cells. In conjunction with their *in vitro* findings, these data suggest that homeostatic proliferation may lead to an interconversion of T_{CM} to T_{EM} cells and that regulators of DNA methylation play a role in the active maintenance of $CD8^+$ T-cell memory subsets.

$CD8^+$ T-cell differentiation is also regulated by active DNA demethylation. TET2 conditional knockout mice (deletion driven by CD4Cre), show no apparent differences in thymocyte or peripheral T-cell subsets, suggesting that TET2 does not play a critical role in T-cell homeostasis. However, TET2 transcripts are rapidly upregulated by TCR signaling and TET2 loss dramatically altered antigen-driven $CD8^+$ T-cell differentiation. We recently demonstrated that the loss of TET2 alters the DNA methylation landscape in activated viral-specific $CD8^+$ T-cells and regulates $CD8^+$ T-cell differentiation in a cell-intrinsic manner. Intriguingly, despite opposing enzymatic function, TET2, like DNMT3A, also represses memory $CD8^+$ T-cell development. Specifically, TET2 deficiency led to enhanced T_{CM} $CD8^+$ T-cell differentiation and promoted secondary recall responses.[36] Enhanced reduced-representation bisulfite sequencing of viral-specific $CD8^+$ T-cells revealed differential methylation largely of intronic regions across the genome. Several known transcriptional regulators of $CD8^+$ T-cell effector versus memory differentiation were associated with differentially methylated regions. Although interesting potential targets have been identified, the disruption of chromatin modifying genes, such as TET2 (and DNMT3A), is likely to impact a wide number of genes that contribute to $CD8^+$ T-cell differentiation. In contrast to the finding that TET2-deficient $CD4^+$ T-cells had decreased T_H1 differentiation and decreased IFNγ production,[78] TET2-deficient $CD8^+$ T-cells had increased IFNγ production, [36] suggesting possible cell-type specific or contextual effects. The phenomenon that DNMT3A and TET2 loss promotes similar (although not identical) cellular phenotypes despite opposite roles in regulating DNA methylation has also been observed in HSCs. [128,129] The explanation for this phenomenon has not yet been elucidated, although recent work has explored the epigenetic underpinning of this observation. In HSCs, Zhang and colleagues examined wild-type, DNMT3A-deficient, TET2-deficient, and double-knockout cells to evaluate the interaction between these epigenetic regulators. The study identified a complex interaction with functional, genomic, and methylation/hydroxymethylation profiling suggesting a combination of independent and interdependent roles.[130] Another study examined methylation patterns in different TET-deficient cell types and found that hypermethylation typically occurred in the active euchromatic compartment, but paradoxically, they noted hypomethylation in the heterochromatic regions.[131] In comparing single TET2 or DNMT3A-deficient HSCs to double-deficient HSCs, both TET2 loss and DNMT3A loss resulted in hypomethylation in the heterochromatin genome-wide. Bioinformatic reanalysis of published chromatin-immunoprecipitation data of tagged DNMT3A1 (a splice variant of DNMT3A) in wild-type and TET1-deficient murine embryonic stem cells[132] revealed that DNMT3A and TET1 occupy mutually exclusive positions in the euchromatin and that DNMT3A1 relocalizes from the heterochromatic

compartment to the euchromatin compartment in TET1-deficient cells,[131] suggesting that DNMT3A and TET proteins may compete for localization to active euchromatic regions.

Importantly, murine studies of T-cell TET2-deficiency share characteristics with human T-cells that have lost expression of functional TET2 protein. Recently, chance disruption of TET2 in a CAR–T-cell infusion product was associated with near clonal expansion of the TET2-disrupted T-cell clone. The appearance of this expanded population correlated with tumor clearance and long-term survival.[133] Similar to murine studies, these cells displayed a T_{CM} phenotype *in vivo*, and experimental knock-down of TET2 in control human T-cells *in vitro* also promoted central memory differentiation.[133]

These data are also in line with studies utilizing the metabolite 2-HG, which inhibits α-ketoglutarate dependent enzymes, including TET family members.[134] This metabolite is made by activated $CD8^+$ T-cells following T-cell activation[135] and treatment of $CD8^+$ T-cells with the S-enantiomer of 2-HG resulted in upregulation of memory-associated molecules CD62L, CD127, and eomesodermin,[135] similar to the *in vivo* phenotype in TET2-deficient $CD8^+$ T-cells. Additionally, S-2-HG treatment resulted in superior persistence and antitumor efficacy of $CD8^+$ T-cells in a model of adoptive cellular therapy. Together, these data raise the possibility that $CD8^+$ T-cells rely on a metabolic mechanism to suppress TET2 activity to promote memory $CD8^+$ T-cell fates.

Given the important role of DNA methylation and DNA methylation-modifying enzymes in T-cell development and differentiation in response to acute antigen exposure and viral challenge, it is not surprising that DNA methylation also impacts $CD8^+$ T-cell responses to chronic infections. In the face of continuous antigen exposure, for instance in the setting of chronic infection or malignancy, $CD8^+$ T-cells undergo a process termed ‘T-cell exhaustion’ (see review by McLane et al.[136]). This state is characterized by expression of multiple inhibitory receptors coupled with progressive loss of effector function, including cytokine production, cytolytic capacity, and ability to proliferate. Exhausted T-cells also have a chromatin landscape that is distinct from other T-cell subsets, with several exhaustion-specific regions having been identified.[137,138] Whole-genome bisulfite sequencing (WGBS) of T-cells during chronic viral infection also revealed that DNA methylation is re-established at specific loci (e.g., *ifng* and *myc*) in exhausted T-cells and that much of this remethylation is dependent on *de novo* methylation, inasmuch as these changes are substantially reduced in DNMT3A-deficient T-cells. Consistent with loss of methylated regions, some features of exhaustion are less severe in the absence of DNMT3A, including cytokine loss and proliferative capacity.[139]

It is well known that immune checkpoint blockade, such as treatment with anti–PD-1 or anti–PL-L1 antibodies, can reverse T-cell exhaustion and lead to clinical responses in a subset of patients with certain cancers; however, for most patients, immune checkpoint blockade does not establish long-term control of their cancer. One contributing factor to this failure is likely the inability of PD-1 blockade to substantially alter the epigenetic landscape of exhausted T-cells as assessed by ATAC-seq[137] and by WGBS.[139] These findings have fueled interest in determining whether modulating epigenetic factors might be beneficial in combination with immune checkpoint blockade. In the case of DNA methylation and

DNMT3A, combination of DNMT3A deficiency and PD-1 blockade therapy, or DNMT3A deficiency and treatment with the DNA methyltransferase inhibitor decitabine prior to PD-1 blockade, reinvigorated exhausted $CD8^+$ T-cell to a greater extent than PD-1 blockade alone in chronic viral infection and murine tumor models, respectively.[139] Ongoing clinical trials will determine whether this approach can improve clinical efficacy in human cancer patients. Early results from one early-phase clinical trial examining the combination of low-dose decitabine with an novel PD-1 blocking antibody versus PD-1 inhibition alone in patients with relapsed/refractory Hodgkin lymphoma found a similar response rate but improved complete response rate (71% vs. 32%) in patients treated with dual DNA methylation inhibition and PD-1 inhibition.[140] Long-term results will be needed to determine duration of response and whether the mechanism functions through enhanced reinvigoration of exhausted antitumor T-cells. Together, these data support a role for DNA methylation in enforcing T-cell exhaustion and may present an attractive therapeutic target to help improve the clinical efficacy of immune checkpoint blockade.

VI. CONCLUDING REMARKS

T-cells are critical mediators of immunity and immunologic memory. Their cell fates are regulated in part through epigenetic mechanisms, including DNA methylation. Recent genome-wide methylation analyses have revealed dynamic alterations in the methylome at various stages of development and differentiation. Additionally, individual DNA modifiers have been implicated in directing different aspects of cell-fate decision, function, and stability. Because T-cells are dysregulated in various disease states (e.g., autoimmune disorders, chronic infections, and cancer), a more complete understanding of how epigenomic programming contributes to these pathologic states is essential. Targeting different DNA modifying enzymes may have potential for modulating immune responses in various clinical settings, including enhancing T-cell regeneration after myeloablative stem-cell transplant and improving immunotherapeutic responses for the treatment of cancer.

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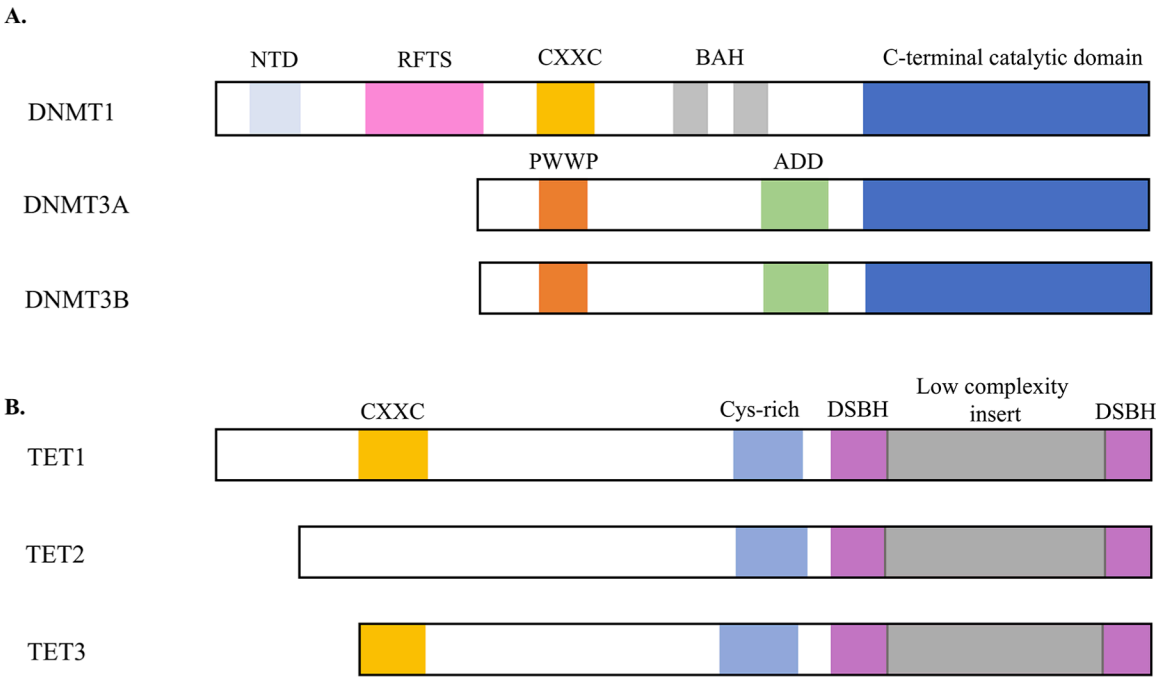

FIG. 1:
Schematic representation of structural domains of DNMT family (A) and TET family (B) members

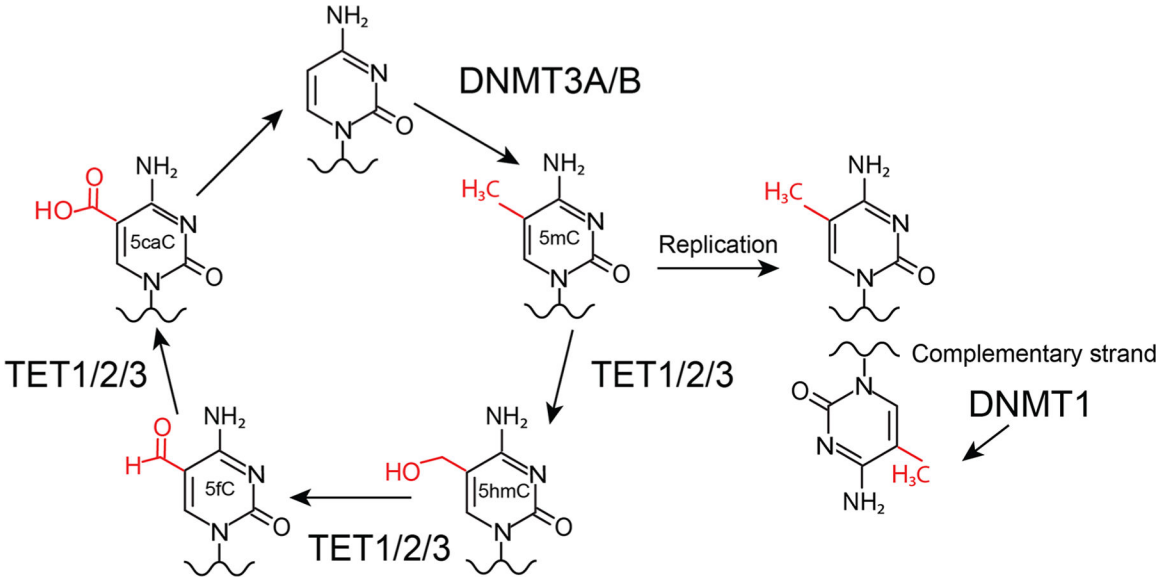

FIG. 2:

DNA methylation and demethylation. DNMT3A/B deposit a methyl group on unmodified cytosine bases to generate 5mC. During replication, DNMT1 methylates daughter strands to maintain 5mC. 5mC can be sequentially oxidized by TET enzymes to 5hmC, 5fC and 5caC. 5fC and 5caC can then be converted to an unmodified cytosine.

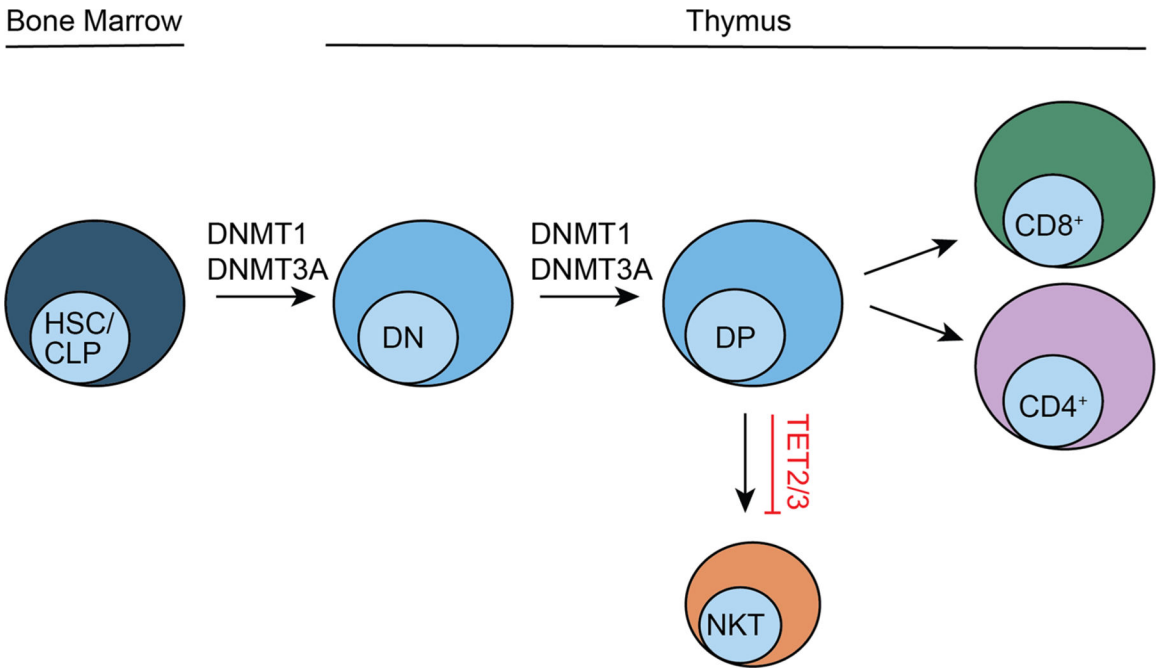

FIG. 3:

DNA methylation enzymes in T-cell development. A schema of T-cell development with the requirement of DNA methylation enzymes noted. Arrows indicate the requirement of the enzyme to proceed through individual developmental transitions. The TET2/3 line indicates suppression of the lineage.

TABLE 1:

Summary of T-cell phenotypes in knockout mice lacking DNMT or TET enzymes

Gene	Cre/mutation type	T-cell phenotype	Reference
DNMT1	Hypomorph	• Decreased lymphoid commitment	Broske et al., 2009[66]
	LckCre	• Profound loss of DP and SP thymocytes; increased apoptosis	Lee et al., 2001[71]
	CD4Cre	• Normal thymocyte development; slight ↓ in $CD44^{hi}$ peripheral $CD4^+$ and $CD8^+$ T-cells; decreased proliferation • Increased cytokine production	
DNMT3A	Germline KO	• Runted, die by 4 weeks • ↓ thymocyte cellularity, partial DN → DP block	Okano et al., 1999[4] Gamper et al., 2009[72]
	CD4Cre	• No thymocyte defects noted • Normal T_H1 and T_H2 *in vitro* differentiation, increased plasticity	Gamper et al., 2009[72]
TET1/TET2	TET1 KO; TET2 CD4Cre	• Increased *FoxP3* promoter and CNS2 methylation • Loss of Treg stability and suppressive function	Yang et al., 2015[106]
TET2/TET3	CD4Cre	• Expansion of NKT17 cells • Fatal antigen-dependent iNKT lymphoproliferative disorder	Tsagaratou et al., 2017[76]
	FoxP3Cre	• Diminished demethylation of *FoxP3* CNS2 and TSDRs of other genes • Impaired long-term stability, increase in ‘ex-Tregs’ • Lethal inflammatory disease	Yue et al., 2019[105]
TET2	CD2Cre	• ↓ T_H1 and T_H17 *in vitro* differentiation Altered cytokines in EAE model	Ichiyama et al., 2015[78]
	CD4Cre	• ↑ central memory $CD8^+$ T-cell differentiation	Carty et al., 2018[36]